Viral and Bacterial Sepsis: Identification and Characterization of Cytokine Profiles and Cell Death Pathways

by

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Abstract

Sepsis and septic shock are the leading causes of death in intensive care units in North America. Approximately 800,000 cases of sepsis are reported every year in the USA and 215,000 will succumb to the disease despite aggressive antibiotic and supportive care [1-7]. The incidence of sepsis is increasing at a rate of 9% per year even with advances in the understanding of the pathophysiology of the disease [1]. Sepsis is commonly induced by bacterial and viral infections. Influenza A is the virus that causes the flu. The most recent pandemic caused by Influenza A (H1N1) demonstrated the potential of this virus to cause severe complications (viral induced sepsis characterized by pneumonia, acute respiratory distress and acute lung injury). This study investigated the inflammatory mediators of sepsis in H1N1 infected adults and from adults with bacterial induced sepsis. For H1N1 infections lung tissue and sera samples were characterized. Lung tissue showed hemorrhage, and interstitial congestion. Cytokine staining of these tissues revealed the presence of TNF-α, IFN-γ and IL-1β primarily from the recruited leukocytes. In addition TUNEL assay revealed apoptosis occurring in these same cell populations. ELISA quantification of sera from H1N1 infected adults also demonstrated high levels of TNF-α, IFN-γ and IL-1β at 113 pg/mL, 49 pg/mL and 26 pg/mL respectively. The sera also were able to induce apoptosis in cultured fibroblasts that was mediated through STAT1 signalling. The sera from bacterial induced sepsis were characterized for the presence of other cytokines and chemokines. TNF-α was consistently detected in all septic samples. Chemokine expression was more variable across the septic samples. The identification of different mediators of inflammation during sepsis will not only increase the basic knowledge about the progression of sepsis but would allow for the development of biomarkers that could better predict the progression of sepsis to more severe or fatal forms and allow for earlier intervention and treatment of these cases.

Key Words: Sepsis, Inflammatory cytokines, H1N1 influenza A, Viral pneumonia, Apoptosis, Immunopathology

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Abbreviations

ACCP	American college of chest physicians
AIF	Apoptosis inducing factor
AKT	Protein kinase B
ALI	Acute lung injury
AP-1	Activator protein one
APAF-1	Apoptotic protease activating factor
ARDS	Acute respiratory distress syndrome
ARDS	Acute respiratory distress syndrome
ATF	Activating transcription factor
ATF-2	Activating transcription factor two
BAX	BCL2-Associated X Protein
Bcl-3	B-cell lymphoma 3-encoded protein
BH	B-cell lymphoma 2-encoded protein homology domain
BIR	Baculovirus inhibitor of apoptosis protein repeat
BMI	Body mass index
b-ZIP	basic region leucine zipper
CARD	Caspase activation and recruitment domain
CC	Cubic centimetre
CD14	Cluster of differentiation fourteen
CD14	Cluster of differentiation 14
c-FLIP	cellular Fas-associated death domain like interleukin 1 beta converting enzyme like inhibitory protein
cGMP	Cyclic guanosine monophosphate
cIAP	Cellular inhibitors of apoptosis protein
CLP	Cecal ligation and puncture
CRM1	Chromosomal maintenance one
CRM1	Chromosome region maintenance 1
CSF-1	Colony stimulating factor 1
DAB	Diaminobenzidine
DD	Death domain
DED	Death effector domain
DFF	Deoxynucleic acid fragmentation factor
DIABLO	Direct IAP-binding protein with low pI
dsRNA	Double stranded ribonucleic acid
EGF	Epidermal growth factor
eIF2α	Eukaryotic initiation factor 2 alpha
ELISA	Enzyme linked immunosorbent assay
ENA-78	Epithelial-derived neutrophil activating protein 78

eNOS	Endothelial nitic oxide synthase
ERK	Extracellular-signal-regulated kinase
ESCRT	Endosomal sorting complexes required for transport
FADD	Fas associated death domain protein
fas	First apoptosis signal
FCS	Fetal calf serum
GAS	Gamma activating sequence
GCSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
GRO	Growth related oncogene
HA	Hemagglutinin
HMGB1	High mobility group box 1
HSP	Heat shock protein
HSS	Human septic serum
I.C.U.	Intensive care unit
I309	Inflammatory cytokine I309
ICAM	Intercellular adhesion molecule 1
IFN	Interferon
IFNGR1	Interferon gamma receptor 1
IGF-1	Insulin-like growth factor 1
IKK	Inhibitor of kappa B kinase
IL-1R	Interleukin 1 receptor
IL-1Ra	Interleukin 1 receptor antagonist
IL-1RAP	Interleukin 1 receptor accessory protein
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
IPS-1	Interferon-beta promoter stimulator 1
IRAK	Interleukin-1 receptor associated kinase
IRF	Interferon regulatory factor
ISGF3	Interferon stimulated gene factor 3
ISRE	Interferon alpha stimulated gene response element
IκB	Inhibitor of kappa b
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KC	Keratinocyte chemoattractant
LBP	Lipopolysaccharide binding protein
LGP-2	Laboratory of genetics and physiology 2
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
M1	Matrix protein1
M2	Matrix protein 2

m5C	5 methyl cytidine
m6A	N-6 methyladenosine
m7G	N-7methylguanosine
MAF	Avian musculoaponeurotic fibrosarcoma oncoprotein
MAL	Myeloid differentiation primary response gene 88 adaptor like
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MCSF	Macrophage colony stimulating factor
MD-2	Lymphocyte antigen 96
MDA-5	Melanoma differentiation associated protein 5
MDC	Macrophage derived chemokine
MEK	Mitogen activated protein kinase kinase
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MIG	Interferon gamma induced monokine
MIP-1α	Macrophage inflammatory protein 1 alpha
MODS	Multiple organ dysfunction syndrome
MYD88	Myeloid differentiation primary response gene 88
NA	Neuraminidase
NAIP	Neuronal inhibitor of apoptosis protein
NEP	Nuclear export protein
NF-κB	Nuclear factor kappa B
NK	Natural killer cells
NLRP3	Nucleotide-binding oligomerization domain like receptor family
nNOS	Neuronal nitic oxide synthase
NO	Nitic oxide
NP	Nucleoprotein
NS1	Non structural protein 1
OAS	2′-5′ oligoadenylate synthetase
PA	Polymerase acidic
$PaCO_2$	Partial pressure of carbon dioxide
PAI-1	Plasminogen activator inhibitor-1
PAMP	Pathogen associated molecular pattern
PB1	Polymerase basic one
PB2	Polymerase basic two
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PECAM-1	Platelet-endothelial cell adhesion molecule-1
PI3K	Phosphatidylinositol 3 kinase

PKR	Protein kinase RNA dependent
Poly (I:C)	Polyinosinic polycytidylic acid
PRD	Positive regulatory domain
PRR	Pattern recognition receptor
Rab11a	Ras related protein eleven a
Raf	Rapidly accelerated fibrosarcoma
Ran	Ras related nuclear protein
RANTES	Regulated on activation normal T cell expressed and secreted
RIG-1	Retinoic acid inducible gene one
RIP1	Receptor interacting protein 1
RNAse L	Ribonuclease L
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
SCCM	Society of critical care medicine
SCF	Stem cell factor
SDF	Stromal cell factor
siRNA	Short interfering RNA
SIRS	Systemic inflammatory response syndrome
SMAC	Second mitochondria-derived activator of caspase
STAT	Signal transducer and activator of transcription
TAFI	Thrombin activatable fibrinolysis inhibitor
TARC	Thymus and activation regulated chemokine
TBK1	TRAF family member-associated NF-kappa-B activator binding kinase 1
TBST	Tris buffered saline with triton X 100
TF	Tissue factor
TGF-β	Transforming growth factor beta
TIR	Toll/interleukin 1 receptor domain
TIRAP	Toll/interleukin 1 receptor domain containing adaptor protein
TLR	Toll like receptor
TMB	Tetramethyl benzidine
TNFR1	Tumor necrosis factor receptor 1
TNF-α	Tumor necrosis factor alpha
TRADD	Tumor necrosis factor receptor associated death domain protein
TRAF6	Tumor necrosis factor receptor associated factor 6
TRAIL	TNF-related apoptosis-inducing ligand
TRAM	Trif related adaptor molecule
TRIF	TIR-related adaptor protein inducing interferon
tRNA	transfer ribonucleic acid
TSST-1	Toxic shock syndrome toxin one
TUNEL	Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling
VCAM	Vascular cell adhesion molecule 1

vRNP	Viral ribonucleoprotein
W.H.O.	World health organization
XIAP	X chromosome-linked inhibitor of apoptosis protein
YB-1	Y-box binding protein 1

Chapter1: Introduction

1.1 Definitions and Epidemiology

Sepsis is a systemic response to an infection [8]. The definition of sepsis was first clarified in 1992 by the American College of Chest physicians and the Society of Critical Care medicine consensus conference for the definition of sepsis. Before this conference the definitions for infection, bacteremia, sepsis, septicemia and septic shock were not precise and used interchangeably [8]. An infection is an inflammation caused by a microorganism present in normally sterile tissue while bacteremia is a condition where viable bacteria are cultured from blood. The term Systemic Inflammatory Response Syndrome (SIRS) is used to describe a non infectious inflammatory state sometimes referred to as sterile sepsis. The SIRS criteria include the following symptoms: 1) fever with a body temperature either greater then 38˚C or hypothermia with a temperature of less then 36˚C, 2) tachycardia with a heart rate of greater then 90 beats per minute, 3) tachypnea of 20 breaths per minute or greater or a $PaCO_2$ of less then 32mmHg, 4) leukocytosis with a white blood cell count greater then 12 000 per cubic millimeter or leukopenia with a count less then 4 000 per cubic millimeter or less then 10% immature white blood cells. Other hemodynamic changes include increased bleeding thrombocytopenia or increased coagulation [8, 9]. These clinical symptoms must occur in the absence of other known causes to be considered SIRS. Based on these criteria sepsis was defined as SIRS with a confirmed infection [8,10]. Sepsis belongs to a continuum of severity of microbial induced systemic inflammation. This continuum commences with an infection which progresses to sepsis. The progression of sepsis continues to a state of severe sepsis. This state is defined as

sepsis with the occurrence of organ dysfunction. The consequences of organ dysfunction include hypoperfusion of tissues, vascular hypotension and the metabolic consequences manifesting as lactic acidosis, oliguria and alterations in mental status. Alterations in mental status include confusion, disorientation, lethargy, agitation and in severe cases coma [9]. Hypotension is defined as a systolic blood pressure less than 90 millimeters of mercury or a decrease of more then 40%. The most common organ dysfunction occurs in the cardiovascular system and respiratory system [11]. Estimates for the incidence of severe sepsis in the I.C.U. was 51 cases per 100 000 people per year. When adjusted for age the incidence was estimated to be at a low of 14 per 100 000 for 20 to 24 years and a high of 58 per 100 000 for 75 to 79 years [11]. Progression from severe sepsis leads to septic shock. This is an acute condition characterized by persistent hypotension in the presence of fluid resuscitation in addition to the existing conditions of severe sepsis. In addition to shock severe sepsis may progress to Multiple Organ Dysfunction Syndrome (MODS). This condition is used to describe altered functioning of multiple organ systems in critically ill patients. MODS is a term that describes a continuum of organ dysfunction. At the most severe end the dysfunction becomes severe enough where the organ can no longer maintain homeostasis and failure of interdependent organ systems begins. Progression of this condition leads to the most severe states of the sepsis continuum which are multiple organ failure and death [8]. These base definitions of sepsis have remained with modifications, additions and clarifications from preceding meetings. In 2001 the addition of testing for biochemical markers of sepsis was included in the diagnosis of sepsis [10]. These include plasma levels of C reactive protein and procalcitonin being greater then 2 standard deviations from normal [10]. In 2004 the Surviving Sepsis Campaign created a goal oriented therapy strategy for the treatment of sepsis [12]. The first goal is fluid resuscitation to occur as soon as sepsis is

recognized. The clinical parameters to achieve by fluid resuscitation are: 1) a central venous pressure between 8 and 12 millimeters of mercury, 2) a mean arterial pressure of 65 millimeters of mercury or greater 3) urine output of at least 0.5mL per kilogram per hour and 4) central venous oxygen saturation of at least 70%. It is recommended that resuscitation occur within the first 6 hours of sepsis diagnosis. Regarding the diagnosis of sepsis it is recommended that 2 cultures be taken before the initiation of antibiotic therapy [12]. The Surviving Sepsis Campaign recommends that antibiotic therapy should be initiated with in the first hour of sepsis. Other recommendations regarding treatments made by the campaign include source control of the site of infection, use of vasopressors when fluid administration fails to adequately increase blood pressure, use of inotropic therapy and steroids [12]. Compliance to these recommendations were tested in an emergency department and found that greater compliance to the resuscitation module gave greater decrease in mortality then low compliance with a 25% reduction in relative risk of mortality [13].

It has been estimated that globally 31.5 million people will develop sepsis, 19.4 million will progress to severe sepsis and 5.3 million people will succumb to sepsis [14]. Sepsis has a large financial impact on health care systems. In the U.S. it accounted for 5.2% of the total aggregate hospital costs or 20.3 billion dollars and was ranked the most costly condition for 2011 [15]. The vast majority of sepsis cases are a result of community acquired infections versus nosocomial infections. In the U.S. community acquired sepsis accounted for two thirds of all sepsis cases [16] and similarly in Finland 58% of sepsis cases were community acquired [17]. Sepsis develops in all age groups but its distribution is bimodal with the greatest mortality occurring in the very young and the very old [18]. The mortality due to sepsis drops dramatically after age one and remains low until age 55 where there is a large rise to age 75 where mortality peaks at

the age group of 85 and older [19]. Males have a higher risk of developing sepsis then females [19]. An English study revealed that sepsis accounted for 4.7% of deaths from 2001 to 2010 with the annual rates of sepsis in males being 20-28% greater then females [18]. For adults the most common sites of infection which led to the development of sepsis are: the lungs, kidneys, urinary tract and abdomen [20]. It has been observed that sepsis in older individuals with co-morbidities of cancer, chronic kidney disease, deep vein thrombosis, diabetes, dyslipidemia, hypertension and obesity contributes an independent increase risk of mortality [20]. Sepsis also leaves lasting side effects on those who survive a septic episode. Older survivors of sepsis have a 13% increase risk of death one year after sepsis compared to the same age group who never developed sepsis and this risk increases with time to a 2 fold increase over non septic individuals 5 years after sepsis [20]. In addition to mortality sepsis has a lasting impact on cognitive function. Elderly survivors of sepsis when compared to other age matched patient populations from the I.C.U. demonstrated greater cognitive impairment. Long term mild cognitive impairment was found in the following areas: attention, verbal fluency, verbal memory and executive function [21].

The host response to an infecting microorganism is to initiate an inflammatory state designed to clear the microbe from the body. If the infection persists and intensifies then the host response results in a hyperinflammatory state defined by SIRS. If the infecting organism is not cleared and sepsis continues the host response enters an anti-inflammatory state which progresses to a state of immunoparalysis. When sepsis is severe and particularly when it occurs in the elderly a long lasting immune system dysfunction manifests itself. This is seen in elderly survivors of sepsis who tend to have longer hospitalization times, increased number of subsequent infections after sepsis that tend to be from opportunistic bacteria, are much more susceptible to developing pneumonia and have a greater risk of developing complications from infections when compared

to elderly non septic individuals [22]. The elderly and the very young are more susceptible to increased mortality and morbidity of sepsis. This may be attributed to the way in which the immune system of each group responds during sepsis. In a murine model of sepsis distinct differences were found between neonatal mice and elderly mice in the way their immune systems reacted to a septic state. Neonatal mice had lower mediators of inflammation in their circulation then the control young mice and were less able to recruit immune cells to the site of infection. Elderly mice produced much higher levels of inflammatory mediators with greater upregulation of reactive oxygen species then controls [23].

1.2 Bacteria and Sepsis

Sepsis can be initiated with a single or localized infection in the host which is termed a nidus of infection [24]. Examples of such infections include but are not limited to abscess, pneumonia, peritonitis, pyelonephritis and cellulitis. The invading organism which include bacteria, parasites or viruses cause a local infection and may circulate in the bloodstream and release components to initiate an inflammatory response [25]. Bacteria cause the majority of sepsis cases, whereas viruses and parasites are reported in approximately 2-4% of the sepsis cases [1]. Gram-positive bacterial induced sepsis is found to be most common with an occurrence of 30-50%. Gram-negative bacterial induced sepsis, which were predominant in the 1970s and 1980s are less frequent with an incidence rate of 25-30% [1]. In addition, polymicrobial infections are identified in 25% of sepsis cases. The inflammatory response can be triggered by the presence of bacterial toxins, either endotoxins or exotoxins. For example, a component of the Gram-negative bacterial outer cell membrane, lipopolysaccharide (LPS) is a classic inducer of the inflammatory cascade [4,25,26]. Examples of bacterial exotoxins include Toxic Shock Syndrome Toxin-1

(TSST-1) and Toxin A released from *Staphylococcus* and *Pseudomonas* species, respectively. Further, structural components of the bacteria may initiate a host response including peptidoglycan and lipoteichoic acid from Gram-positive bacterial cell wall [1,6,24]. More recently, studies have demonstrated that pure bacterial DNA and RNA can stimulate a host response [24].

1.3 Initiating Molecules of the Inflammatory Response

The immunological host response to invading organisms includes innate and adaptive immunity [7,27,28]. The innate response is the body's first line of defense and is therefore responsible for the recognition of pathogens for subsequent elimination [7,27]. Immune cells including neutrophils, monocytes and macrophages are key components of the host response. These cells are capable of recognizing structural components or products from bacteria including LPS, peptidoglycan and lipoteichoic acid [27,29-33]. These products vary among different strains or species; however, all microbes possess an invariable pathogen-associated molecular pattern (PAMP) that can be recognized by pattern recognition receptors (PRRs) on innate immune cells [31,34,35]. Consequently, PRRs will specifically activate signalling pathways to mediate the inflammatory response by releasing pro-inflammatory mediators.

1.4 Gram-Negative Bacteria

Previously, the most common infections leading to the development of sepsis were initiated from Gram-negative bacteria [1]. The PAMP for this type of bacteria is LPS since it is the principal component of the bacterial outer cell membrane [6,30,36,37]. LPS is an amphiphilic macromolecule that is composed of three major elements [24,26]. These include the

polysaccharide side chain, the phospholipid and the lipid A; the last component being the most toxic element of the endotoxin [24]. The lipid A moiety was shown to reproduce many clinical and metabolic manifestations observed during septic shock [24]. The presence of LPS in the body, even at low levels, activates monocytes and macrophages through recognition of the invariant pattern of the lipid A portion [30]. Initially, LPS forms a complex with a plasma protein called lipopolysaccharide binding protein (LBP) [24,30,32,37]. The LBP catalyzes the transfer of the LPS to a membrane bound receptor termed CD14 on the surface of monocytes and macrophages or to a soluble CD14 molecule found in the plasma for activation of CD14 negative cells such as endothelial cells [24,32,36]. CD14 along with Toll-like receptors (TLRs) belong to the family of PRRs. Subsequent to LPS binding to CD14, LPS is transferred to TLR 4 [24]. TLR 4 belongs to a family of ten members of which are characteristically identified by the presence of leucine-rich repeat (LRR) domain in the extracellular region [27-29]. In addition, TLRs possess a cytoplasmic region that is homologous to the interleukin-1 receptor (IL-1R) referred to as the Toll/IL-1 receptor (TIR) domain [31]. Consequently, the signalling pathways between the pro-inflammatory cytokine IL-1 and the TLRs possess similarities. However, the binding of LPS to TLR 4 is not sufficient to stimulate an adequate inflammatory response. Shimazu *et al* have shown that an adaptor protein termed MD-2 interacts with TLR 4 on the cell surface which is required in order to enhance the inflammatory response. Therefore, LPS recognition by immune cells necessitates the formation of large complex whereas TLR 4 will transduce the signal downstream to activate transcription factors such as NF-κB and AP-1 that modulate the host response [32,38]. TLR 4 is not exclusive to LPS recognition. It is also able to bind to other ligands such as Taxol, heat shock proteins (HSP 60 and 70) as well as host extracellular matrix components (fibronectin, hyaluronic acid and fibrinogen) [28].

1.5 Gram-Positive Bacteria

Gram-positive bacteria components such as peptidoglycan, a large polymer that provides most of the strength and rigidity of bacterial cell walls and teichoic acid, a diverse group of polymers found in the cell wall or cell membrane are also able to initiate the inflammatory response. The presence of these structural components will be recognized by another member of the TLR family, more specifically, TLR 2 [27-29]. Similarly to LPS, peptidoglycan and lipoteichoic acid will bind to the TLR receptor in a CD14 dependent manner [33]. However, the accessory protein MD-2 is unique to TLR 4 recognition and is not required for the activation of the inflammatory response through TLR 2 [28]. Other ligands distinguished by TLR 2 include lipoproteins, lipoarabinomannan (mycobacteria), atypical LPS and porins (*Neisseria*) [28]. In addition, researchers have shown that TLR 2 is able to cooperate with other members of the TLR family to recognize other microbial components. More specifically, TLR 1 and TLR 6 are functionally associated with TLR 2 and are able to discriminate between other ligands such as tri-acyl lipopeptides and di-acyl lipopeptides, respectively [28]. Upon ligand recognition by TLR 2, a signalling pathway will be initiated to activate transcription factors such as NF-κB and AP-1 that modulate the inflammatory response.

1.6 Bacterial DNA

Another potent activator of the inflammatory response is bacterial DNA. Bacterial genomic DNA contains unmethylated CpG dinucleotides [28,39,40]. In contrast, mammalian DNA has a low level of CpG motifs and is usually found to be methylated. As such, innate immune cells are able to discriminate between their own DNA and prokaryotic DNA. Thus, CpG motifs are yet another example of a PAMP by which TLRs are able to recognize. In this case, TLR 9 has been

identified as the family member responsible for the stimulatory effects of the innate response in the presence of bacterial genomic DNA [28,29,31,41]. Unlike TLR 1, 2 and 4 which are found to be expressed on the cell surface, TLR 9 has been identified as being in the endosome [28,39]. Studies have demonstrated that CpG DNA enters the cell by endocytosis and that TLR 9 is expressed on the surface of the endosome [28]. Consequently, TLR 9 is able to initiate a signalling pathway to activate the AP-1 and NF-κB transcription factors.

1.7 Double-stranded RNA

The innate response has also evolved to recognize the presence of viral components such as double stranded RNA (dsRNA). dsRNA may be produced during the viral replication cycle [28]. It has been demonstrated to be a strong inducer of type I interferons such as interferon alpha (IFN-α) and interferon beta (IFN-β) [28]. Furthermore, dsRNA is able to promote the transcription of interferon inducible genes and thus stimulate the host response. A synthetic dsRNA called polyinosinic-polycytidylic acid [poly (I:C)] has been shown to exhibit similar activity to dsRNA [28,41]. The stimulatory effects of dsRNA were thought to originate from a protein termed dsRNA-dependent protein kinase (PKR). However, previous studies have indicated that other molecules are involved in viral recognition. More specifically, TLR 3 is able to respond to the presence of dsRNA and elicit an immunological response through NF-κB activation [28,29,31].

1.8 Bacterial RNA

Bacterial RNA can also elicit an immunological response. More recently, the notion that TLRs can distinguish between mammalian and bacterial RNA through nucleoside modifications was

investigated [42]. The majority of cellular RNA is comprised of ribosomal RNA (rRNA) and is modified in mammalian cells versus bacterial cells. For example, human rRNA possesses approximately ten-fold and twenty-five fold the amount of pseudoouridine and 2'-O-methylated nucleosides respectively when compared to bacterial rRNA [42]. Another component of cellular RNA is the transfer RNA (tRNA). Humans minimally possess 25% of tRNA modified nucleosides whereas bacterial tRNA is rarely modified. Similarly, bacterial messenger RNA (mRNA) does not contain nucleoside modifications as compared to human mRNA [42]. Typical examples of human mRNA nucleoside modifications include 5-methylcytidine (m5C), N6-methyladenosine (m6A), inosine, 2'-O-methylated nucleosides and N7-methylguanosine (m7G) [42]. Results from *in vitro* studies indicate that unmodified nucleosides are recognized by immune cells through TLR 3, 7 and 8 [42].

1.9 TLR Receptor Signalling Pathways

As mentioned previously, a variety of stimuli are able to initiate a host response. These foreign antigens are specifically recognized by different members of the TLR family where they transduce the signal to downstream effectors. The most common effectors are the transcription factors AP-1, NF-κB and IRF [29]. Despite the fact that all the TLR members are able to activate NF-κB and MAP kinases with the exception of TLR3, the resulting gene expression profile varies among the individual TLR [29]. A possible explanation for this specificity is the adaptor proteins being recruited by the TLRs.

1.9.1 MyD88-dependent Pathway

The most common intracellular signalling pathway utilized by all TLRs except for TLR3 is the activation of NF-κB and MAP kinases through the recruitment of the adaptor protein called

MyD88 [27,29]. The signalling pathway will begin in the common TIR domain on the cytoplasmic surface of each TLR. A homophilic interaction between the TIR domains of the TLR and the C-terminal portion of the adaptor protein MyD88 will occur [27,38]. In turn, the N-terminus of MyD88 contains a death domain which will recruit a death domain containing serine/threonine kinase called IL-1 receptor-associated kinase 4 (IRAK-4) [35,38]. Consequently, IRAK-4 becomes activated and phosphorylates IRAK-1. Activated IRAK-1 recruits TNF receptor associated factor 6 (TRAF6) which ultimately leads to the activation of the following kinases: 1) Inhibitor of NF-κB kinase (IKK) complex; 2) p38; and 3) Jun N-terminal kinase (JNK) [14,20]. The IKK pathway eventually leads to the activation of the transcription factor NF-κB whereas the p38 and JNK mitogen-activated protein kinase signalling pathways converge to activate the transcription factor AP-1 [27,38].

1.9.2 MyD88-independent Pathway

In addition, a MyD88-independent pathway has been identified. MyD88 deficient cells are able to activate NF-κB and JNK in response to LPS stimulation. However, a delayed reaction is observed [28]. In this pathway, a novel adaptor protein termed TIR domain-containing adaptor protein (TIRAP)/MyD88-adaptor-like (Mal) was discovered. TIRAP/Mal has a C-terminal TIR domain but lacks the N-terminal death domain observed in MyD88 [28-30,35]. Recruitment of IRAK-2 by TIRAP/Mal occurs and eventually leads to NF-κB and JNK activation [29,33]. Furthermore, stimulation of MyD88 deficient cells by LPS also activates Interferon-Regulatory Factor-3 (IRF-3) [28,30]. Consequently, IRF-3 activation induces the expression of many IFN-inducible genes.

1.9.3 Other Adaptor Proteins

In addition to MyD88, other adaptor proteins have recently been identified that contribute to the specificity of TLRs. For instance, TLR4 and TLR3 are both able to recruit a novel protein called TIR-related adaptor protein inducing interferon (Trif) [30,41]. Trif signals through an IKK-like kinase called TBK-1 which is required for the phosphorylation and activation of the transcription factor IRF3 and IRF7 [35,40]. In addition, another adaptor protein was shown to be involved in TLR4 signalling. This protein named Trif-related adaptor molecule (Tram) has been shown to act as a bridging adaptor for the subsequent recruitment of Trif [29]. Therefore, this distinct individual TLR signalling may explain the various biological responses to different stimuli. Furthermore, PAMPs have been shown to activate transcription factors independent of the TLR family. For instance, LPS has been shown to activate another family of transcription factors, namely, the Signal Transducer and Activator of Transcription (STAT) [28]. STAT-1 deficient mice were subjected to LPS and a reduction of IFN-regulated genes was observed. This data indicates a potential role of STAT-1 in the response to LPS [28].

In summary, the recognition of PAMPs by innate immune cells will activate complex signalling pathways which ultimately lead to the activation of transcription factors such as NF-κB, AP-1, STAT and IRF. Accordingly, these transcription factors will induce the inflammatory response through the expression of inflammatory mediators such as cytokines, chemokines, cell-adhesion molecules and inducible nitric oxide synthase (iNOS) [34].

1.10 Transcription Factors

1.10.1 NF-κB

Nuclear Factor-κB (NF-κB) proteins play a significant role in the host response against pathogen invasion. Recent studies in animal models of sepsis and septic shock have identified NF-κB as having a key role in the pathophysiology of sepsis [42]. NF-κB is a family of structurally related proteins that form homodimers and heterodimers with various members of the NF-κB/Rel family [42,44]. Such members include NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), RelB and C-Rel. NF-κB1 (p50) and NF-κB2 (p52) are synthesized as precursor p105 and p100, respectively [34, 43-45]. Members of this family can associate to form a variety of dimers which have distinct properties and are able to differentially regulate gene expression. The most common association is the heterodimer p50/p65 [43]. A common characteristic of this family is the presence of a 300 amino acid Rel homology domain. The Rel domain is important for dimerization, as well as DNA binding. It is also responsible for binding to a family of inhibitory proteins called Inhibitors of NF-κB (IκB). In unstimulated cells, NF-κB subunits are bound to IκB proteins. These proteins include IκBα, IκBβ, IκBγ, IκBε, IκBζ, Bcl-3, p105 and p100 [34,43]. These inhibitory proteins can interact with NF-κB proteins though their characteristic ankyrin repeat domain. This interaction masks the nuclear localization sequence on NF-κB proteins and thus, serves as a regulatory mechanism by sequestering the transcription factor to the cytoplasm [43,44]. The IκB proteins will be phosphorylated by IKKs and thus signalled for ubiquitination and degradation upon NF-κB activation by LPS or cytokines [43-46]. Therefore, the NF-κB proteins can translocate to the nucleus and bind to its cognate regulatory element to modulate gene expression.

NF-κB broadly regulates biological functions including the regulation of inflammatory mediators. In addition, it has been found to play a pivotal role in the regulation of cell proliferation and apoptosis [43,48]. Under normal conditions, NF-κB generates pro-survival signals by modulating the anti-apoptotic genes such as inhibitors of apoptosis-1 and -2 (IAP-1 and IAP-2), the X chromosome-linked inhibitor of apoptosis protein (XIAP) and the cellular Fas-associated death domain-like IL-1β-converting enzyme (FLICE)-like inhibitory protein (c-FLIP), an inhibitor of caspase 8 [43,44]. However, if the activity of NF-κB is deficient or inhibited, the cell will undergo apoptosis. In contrast, recent findings have indicated that activation of NF-κB can lead to apoptosis through the expression of pro-apoptotic genes such as Fas ligand and c-Myc. A plausible explanation for this contradiction is the fact that NF-κB may switch from one response to another depending on the time and the stimulus [43].

NF-κB also plays an important role in adaptive and immune responses. For example, NF-κB is required for hematopoiesis and for myeloid and lymphoid cell differentiation in the adaptive response [43]. Similarly, during innate immunity, NF-κB plays a pivotal role in the development and maturation of hematopoietic cells such as macrophages and neutrophils [43]. More importantly is NF-κB's role in the inflammatory response. NF-κB modulates the expression of many pro-inflammaotory mediators such as cytokines, chemokines, cell-adhesion molecules and iNOS [34,44]. An increased transcriptional level of pro-inflammatory mediators is thought to be the hallmark of the pathophysiology of sepsis [43]. Previous studies have implicated NF-κB in the pathogenesis of septic shock. Approximately 200 genes that have been implicated in the pathophysiology of sepsis and septic shock contain NF-κB regulatory elements in their promoters [43]. NF-κB regulated genes include: cytokines (TNF-α, IL-1β); chemokines (macrophage inflammatory protein (MIP)-1α, regulated on activation normal T cell expressed

and secreted (RANTES); adhesion molecules (ICAM-1, VCAM-1, E-selectin); and enzymes (iNOS, phospholipase A2). Interestingly, the activity of NF-κB seems to correlate with the severity of the disease [34,43].

1.10.2 AP-1

The Activator Protein 1 (AP-1) is a complex of DNA binding proteins that belong to the basic region-leucine zipper (bZIP) family [47-49]. The basic leucine zipper is required for DNA binding as well as for interaction with other family members to form dimers. The formation of dimers is essential for recognition and binding to the palindrome DNA promoter sequence [47,49]. The AP-1 complexes can form homodimers with Jun family members (c-Jun, v-Jun, Jun-B and Jun-D) or heterodimers with Fos family members (c-Fos, Fos-B, Fra-1, or Fra-2) [47-49]. The properties of the basic leucine zipper of Fos family members does not allow homodimerization [47]. In addition, Jun and Fos can form heterodimers with other bZIP family members such as Activating Transcription Factor (ATF) and Maf [48]. Therefore, the subunit composition of the AP-1 complexes can vary significantly which leads to differential gene expression [49].

The heterodimer c-Jun/c-Fos is expressed in the majority of cell types [48]. However, a variety of stimuli can promote dimerization. These stimuli include cytokines (TNF-α, IL-1, IFN-γ), growth factors, serum, oncoproteins and cellular stress [48,49]. The induction of AP-1 activity upon these stimuli indicates that AP-1 is involved in growth regulation as well as inflammatory and innate immune responses [49]. Several stimuli dependent mechanisms exist that lead to AP-1 activation. For example, growth factors will induce AP-1 activity through a subfamily of the mitogen-activated protein kinases (MAPKs) called the extracellular signal-regulated kinases

(ERK) [47,49]. Upon pro-inflammatory cytokine stimulation, the activation of AP-1 will most likely utilize two other MAPKs subfamilies called JNK and p38 [49]. The p38 MAPKs are involved in *fos* and c-*jun* gene induction [49]. Subsequently, JNK will bind and phosphorylate c-Jun thereby increasing the transcriptional activity. The Fos/Jun heterodimers subsequently bind to AP-1 promoter sites and regulate the gene expression. AP-1 regulates the expression of numerous cytokines and tissue-destructive enzymes such as collagenase [48]. Similar to NF-κB, AP-1 has also been shown to induce the expression of apoptotic and survival related genes. For example, AP-1 has previously been shown to induce the expression of the proapoptotic gene Fas-ligand (FasL) [49]. Cyclin D is another important gene regulated by AP-1 and may play a role in proliferation and cell death [49]. Cyclin D primarily induces cell proliferation, however, when overexpressed, cyclin D contributes to apoptosis in certain cell types [49]. An additional target gene for induction of apoptosis by AP-1 is p53. High levels of c-Jun repress p53-mediated p21 induction thereby allowing p53 activity to promote apoptosis [49]. In summary, AP-1 is a transcription factor that is activated in response to growth factors and cytokines that mediates gene expression involved in inflammatory and immune response as well as cell survival and apoptosis.

1.10.3 STAT

The Signal Transducers and Activators of Transcription (STAT) are cytoplasmic proteins that belong to a family of seven members [50,51]. The STAT members are STAT 1, STAT 2, STAT 3, STAT 4, STAT 5a, STAT 5b and STAT 6 [37-39]. A wide variety of extracellular signalling polypeptides can activate STAT transcription factors. Examples of STAT activators are the epidermal growth factor (EGF), platelet-derived growth factor (PDGF), colony-stimulating factor-1 (CSF-1), the pro-inflammatory cytokines interferon alpha (IFN-α), interferon beta (IFN-

β), interferon gamma (IFN-γ) and interleukins (IL) 2 to 7, 10 to 13, and 15 [50,52,53]. During cytokine signalling, the extracellular ligand binds to its cognate cell surface receptor and activate the Janus protein tyrosine kinases (JAKs) [54,55]. The JAKs autophosphorylate and phosphorylate tyrosine residues on the cytoplasmic domain of the surface receptor [52]. Consequently, the STAT proteins are recruited to the receptor through their src-homology 2 (SH2) domains which recognize and bind to phosphorylated tyrosines [50,51]. The bound STAT proteins will in turn be tyrosine phosphorylated by JAKs. Once phosphorylated, the STAT proteins will form homodimers or heterodimers depending on the ligand and dissociate from the receptor. Once dissociated, the dimer translocates to the nucleus and regulate gene expression [50,52,55,56]. Conversely, non-cytokine activators such as EGF, PDGF and CSF-1 are able to activate the STATs directly through their receptor tyrosine kinases, and therefore, do not require the JAK activation.

Differential gene expression will occur due to the various dimer combinations. The majority of the STAT proteins form homodimers, however, heterodimers such as STAT 1-STAT 2 as well as STAT 1-STAT 3 are able to form in response to cytokines [50,52]. Subsequently, the dimers translocate to the nucleus and bind to specific promoter sequences and mediate gene expression of target genes. The dimerized STATs bind to a specific sequence called the gamma-activating sequence (GAS) [52]. The GAS is present in IFN-γ inducible genes including transcription factors (IRF-1, IRF-8), adhesion molecules (ICAM) and chemokines (IFN-γ-induced monokine [MIG]) [42].

However, if STAT1-STAT2 heterodimer form a complex with a DNA binding adaptor protein called p48, also known as IRF9 and ISGF3γ, the complex will preferentially bind to an interferon alpha-stimulated gene response element (ISRE) [52]. The ISRE is present in a variety of genes

including; transcription factors (IRF-1, IRF-2); cytokines (IL-6, IL-2); enzymes (2'-5' oligoadenylate synthetase, iNOS, PKR), chemokines (chemokine ligand 5 [CCL5]) and adhesion molecules (ICAM-1, VCAM-1) [57]. In summary, the Stat family members broadly regulate cell cycle, cell adhesion, apoptotic and iNOS genes which are found to be implicated in the pathogenesis of sepsis.

1.10.4 IRF

Interferon Regulatory Factor (IRF) is a family of transcription factors comprised of nine members; IRF-1, IRF-2, IRF-3, IRF-4, IRF-5, IRF-6, IRF-7, IRF-8 and IRF-9/p48/ISGF3γ [54,58]. All members contain a characteristic DNA binding motif in the N-terminal region to which they bind to ISRE, interferon consensus sequence (ICS) or interferon regulatory factor element (IRF-E) also known as positive regulatory domain (PRD) I and III [59-62]. In general, the expression of IRF members is constitutive and is activated by post translational modifications such as phosphorylation or acetylation. Certain IRF members can be induced in response to cytokines such as IFNs, TNF, IL-1, IL-6, dsRNA and viral infection [54]. The IRF family members differentially regulate the transcription of many genes including: cytokines (IFN-α, IFN-β, IL-18), chemokines (RANTES, IP-10) and enzymes (PKR, iNOS, 2'-5'OAS) [42,44]. These genes are directly involved in the innate immune response against viral infections [54]. Furthermore, IRFs are involved in regulating processes such as cell growth inhibition, hematopoietic differentiation, cytokine signalling and apoptosis [58,59].

In summary, transcription factors such as NF-κB, AP-1, STAT and IRF are activated in response to pathogen invasion. Consequently, these proteins will modulate the expression of hundreds of

genes involved in biological processes such as inflammatory and innate response, cell cycle and apoptosis.

1.11 Inflammatory Mediators

The inflammatory response is designed to eradicate the microorganism and allow for potential tissue repair. However, an excessive pro-inflammatory response may lead to tissue injury and organ dysfunction [7]. Therefore, the host response should be tightly regulated in order to maintain a balance between a protective inflammatory response and one that is detrimental to the individual. During sepsis, this balance is shifted towards an overwhelming pro-inflammatory response and thus leads to cellular dysfunction, tissue damage, organ dysfunction and potentially, death [7]. This next section will describe important pro-inflammatory mediators which have been shown to play a role in the pathogenesis of sepsis. Cytokines are important mediators of the inflammatory response and also serve to amplify the host response. Of these, tumor-necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), and interferon gamma (IFN-γ) have been shown to be elevated in serum from septic patients [63,64].

1.11.1 Tumor Necrosis Factor-alpha

Tumor necrosis factor-alpha (TNF-α) is a potent cytokine synthesized by a variety of cell types which include macrophages, monocytes and lymphocytes in response to inflammation or injury [64-66]. TNF-α plays a pivotal role in the immune response by modulating the expression of other cytokines, chemokines and cell-adhesion molecules in order to promote the extravasation and attraction of leukocytes to the site of infection [66]. However, systemic and chronic production of TNF-α which occurs during sepsis, can have harmful effects on various tissues and organs possibly resulting in death [65]. For example, TNF-α induces fever (via IL-1β) and

stimulates the acute-phase response (via IL-6) [64,65]. Further, TNF-α activates the coagulation cascade and induces myocardial depression as well as vascular leakage when present at high concentrations [65]. This finding was confirmed through the administration of TNF-α to animals in a variety of sepsis experiments. The physiological response of the animals to TNF-α closely mimicked the clinical manifestations observed during septic shock including cardiovascular collapse, hypotension, intravascular coagulopathy and myocardial suppression [65]. Elevated levels of circulating TNF-α have been reported in patients with sepsis and septic shock [64]. In addition, a correlation was established between elevated levels of TNF-α and the severity or poor outcome of septic patients [64].

TNF-α exerts its cellular effects when the soluble trimer binds to its cell-surface receptor [64]. There are two distinct cell surface receptors that have been identified for TNF-α: type I or TNFR1 and type II or TNFR2 which preferentially bind to soluble and membrane bound TNF-α, respectively [64,66-69]. The majority of cell types constitutively express TNFR1 whereas TNFR2 is tightly regulated. The majority of the biological actions of TNF-α will be initiated through TNFR1 [64,65,67,68]. Binding of TNF-α to its receptors will ultimately lead to the activation of the transcription factors AP-1 and NF-κB. TNF-α can also initiate a caspase signalling cascade leading to apoptosis [66,67]. Recent findings of the formation of two distinct signalling complexes have provided a plausible explanation in the selection of either the pro-survival or apoptosis processes [70].

The TNF-α homotrimer will bind to TNFR1 which will initiate receptor trimerization. This conformational change will result in the recruitment of the adaptor protein called TNFR1-associated death domain protein (TRADD) through TNFR1's cytoplasmic death domain (DD) [70]. Once TRADD is recruited to TNFR1, it will serve as a platform to recruit subsequent

proteins. These secondary proteins are receptor-interacting protein 1 (RIP1), TNF-receptor-associated factor 2 (TRAF2) and a member of the Inhibitors of Apoptosis family called cIAP1 [70]. Hence, TNFR1, TRADD, RIP1, TRAF2 and cIAP1 will form a complex termed Complex I which is formed early after receptor activation. Consequently, RIP1 will recruit a macromolecule called the "signalosome" [70]. The signalosome is composed of three major proteins; inhibitor of NF-κB kinase 1 (IKK1), inhibitor of NF-κB kinase 2 (IKK2) and inhibitor of NF-κB kinase γ (IKKγ) also called NEMO [71]. These kinases are able to phosphorylate the inhibitors proteins of NF-κB (IκB). NF-κB is tightly regulated by residing in the cytoplasm when associated with IκB. Once phosphorylated, IκB will be ubiquitinated and degraded by the proteosome [71]. Once IκB is degraded, the liberated NF-κB monomers dimerize and translocate to the nucleus where they bind to their cognate regulatory elements and modulate the expression of target genes.

In contrast, a few hours after TNFR1 activation or possibly after receptor internalization, the TRADD-based complex can dissociate from the receptor [70]. Once dissociated, this allows for the formation of Complex II through the recruitment of Fas-associated death domain protein (FADD) and Caspase 8 [70]. This complex will promote cell death which will be discussed in further details in the following sections.

In summary, TNF-α is a primary mediator of the immune response through the activation of transcription factors such as NF-κB and AP-1. Hence, TNF-α is able to amplify the host response by modulating the expression of inflammatory mediators.

1.11.2 Interleukin-1 Beta

Interleukin-1β (IL-1β) is a pro-inflammatory cytokine that is produced mainly by mononuclear phagocytes and neutrophils when triggered by bacterial products such as LPS or by cytokines such as TNF-α [7,64]. IL-1β is synthesized as a 31 kDa precursor and is proteolytically cleaved by interleukin-1 converting enzyme (ICE), also known as caspase 1, into an active 17 kDa fragment [72]. IL-1β plays a pivotal role in the regulation of innate immunity by stimulating the expression of genes such as chemokines, cytokines and cell surface receptors. Similar to TNF-α, the biological effects of IL-1β are dependent on its concentration. At low levels, IL-1β will primarily act on endothelial cells to increase surface molecules for leukocyte recruitment as well as promote a coagulation response [7]. In high quantities, IL-1β will exert systemic effects such as fever, acute phase plasma protein production, leukocytosis and thrombocytosis [73]. Previous studies have indicated that IL-1β may play a role in the pathogenesis of sepsis [63]. For example, deletion of the IL-1 receptor in mice resulted in increased resistance to endotoxin lethality. Furthermore, increased levels of IL-1β have been documented in spontaneous human sepsis and septic shock [48]. Elevated levels of IL-1β have been correlated with increased mortality. TNF-α, and IL-1β are found to be the primary mediators of septic shock [64,73].

IL-1β binds to two membrane receptors, the IL-1 receptor 1 (IL-1RI) and the IL-1 receptor 2 (IL-1RII). However, only IL-1RI transduces the signal to downstream effectors whereas IL-1RII acts as a decoy [74]. Upon IL-1β binding to IL-1RI, IL-1RI will recruit an accessory protein called IL-1RAP through heterodimerization of their common cytoplasmic TIR domains [72]. Consequently, signalling will occur through the recruitment of MyD88, IRAK1, IRAK4 and TRAF6 adaptor proteins as previously described for TLR MyD88 dependent signalling pathway

[72]. Therefore, IL-1β will induce the activation of NF-κB and MAPK pathways and amplify the host inflammatory response.

1.11.4 Interferon Gamma

Interferon-γ (IFN-γ), also known as type II interferon, is a pro-inflammatory mediator produced by activated natural killer (NK) cells and T-helper cells [54,63]. Cytokines such as TNF-α, IL-2 and IL-12 will enhance the transcription of IFN-γ [57,75]. IFN-γ is involved in biological processes which include antiviral and immune responses, cell growth and differentiation and apoptosis [54,57]. The main role of this pro-inflammatory cytokine is to activate mononuclear phagocytes [75]. IFN-γ initiates signalling pathways that culminate in the synthesis of enzymes implicated in the respiratory burst and thus allows for macrophages to phagocytose the invading microbe [75]. In addition, IFN-γ activates neutrophils which lead to an up-regulation of the respiratory burst. Also, vascular endothelial cells are activated by IFN-γ thereby stimulating lymphocyte adhesion and extravasation. Further, IFN-γ increases the expression of Class II Major Histocompatibility Complex (MHC) on the surface of a wide variety of cell types [54,75]. As a result, there is enhanced antigen presentation on T cells which amplifies the recognition phase of the immune response. IFN-γ has also been implicated in the pathogenesis of septic shock. *In vitro* administration of LPS, TNF-α, IL-1β and IFN-γ has been shown to have synergistic effects mimicking the adverse hemodynamic effects observed during septic shock [63].

IFN-γ binds to its cognate cell surface receptor which is composed of two subunits; the IFN-γ ligand-binding IFNGR-1 subunit and the accessory IFNGR-2 subunit [54]. Upon ligand binding, heterodimerization of the receptor subunits will occur and allow for subsequent signal

transduction through the JAK-STAT pathway. IFNGR-1 subunit will interact with JAK-1 whereas IFNGR-2 interacts with JAK-2 [54]. These kinases will recruit latent cytoplasmic STAT 1 and allow for homodimerization. Subsequently, the dimer will translocate to the nucleus and bind to the GAS sequence and regulate the expression of target genes [54]. Therefore, IFN-γ amplifies the inflammatory response through STAT activation and expression of IFN-induced proteins.

1.11.5 Other Important Cytokines and Mediators

Transforming growth factor-beta (TGF-β) is an important cytokine which has been recently implicated in the pathogenesis of septic shock. TGF-β is expressed in nearly all cell types and plays a pivotal role in the normal development of embryonic tissues including the heart. In adults, TGF-β has been implicated in tissue repair and fibrosis. During sepsis, TGF-β counteracts the actions of TNF-α and IL-1β in the acute phase of the inflammatory response [64]. The synergistic combination of TNF-α and IL-1β has been shown to partially mediate myocardial depression that occurs during septic shock [76]. A recent study demonstrated a dose dependent reversal of TNF-α and/or IL-1β induced myocardial contraction depression by TGF-β [77].

Interleukin 6 (Il-6) is a cytokine that is described as having both pro- and anti-inflammatory effects [78]. IL-6 is synthesized by activated monocytes, macrophages, vascular endothelial cells, fibroblasts and activated B and T lymphocytes [63-65]. IL-6 levels are influenced by other cytokines, including TNF-α and IL-1 in humans [78]. The biological actions of IL-6 ar manifested in hepatocytes, B cells and other cell types. IL-6 induces the production of acute-phase hepatic proteins [63-65] and as a result, there is an augmentation in circulating fibrinogen and other proteins that are involved in the transportation and coagulation system. In addition, IL-

6 functions as a growth and differentiation factor for activated B cells [65]. IL-6 does not seem to be directly involved in stimulating tissue injury, however, IL-6 plasma levels are steadily elevated in patients with sepsis [63,65,78]. Also, a correlation has been established between the elevated levels of IL-6 and poor outcome in sepsis patients [78] and this implies that IL-6 may be an important marker of systemic inflammation [65].

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine which is ubiquitously produced in organs including the heart [79]. Upon exposure to a variety of stimuli including endotoxin, toxic shock syndrome toxin 1 and cytokines (TNF-α and IFN-γ), MIF is produced by monocytes and macrophages [63]. MIF will amplify the host response by increasing the production of other pro-inflammatory mediators. Elevated levels of MIF have been documented in patients in septic shock [64] and previous studies have identified MIF as a myocardial depressant factor implicated in LPS induced cardiac dysfunction [79].

High mobility group box 1 protein (HMGB1) has also been implicated in the pathogenesis of sepsis and more specifically, Gram-negative sepsis. HMGB1 was initially identified as a nuclear DNA binding protein and transcription factor [80] and has been recently characterized as a cytokine that binds to cell surface receptors including TLR2 and TLR4. HMGB1 plays a role in the following processes: fever, epithelial barrier dysfunction, chemotactic cell movements, release of pro-inflammatory cytokines and acute inflammation [80,81]. HMGB1 is expressed by macrophages after exposure to early pro-inflammatory cytokines which include TNF-α and IL-1 [64]. Administration of LPS to mice resulted in high levels of HMGB1 in the serum 8-32 h post exposure. Also, elevated levels of HMGB1 were noted in patients succumbing to septic shock [64,82].

In summary, cytokines are rapidly produced in response to invading pathogens by mononuclear phagocytes and other cell types. These cytokines will bind to their cognate cell surface receptors and stimulate the transcription of genes involved in the host response.

1.11.6 Inducible Nitric Oxide Synthase

Nitric oxide (NO) is a cellular mediator that is produced in the endothelium and other cell types [83]. NO is a potent vasodilator that mediates the acute inflammatory response by increasing the blood flow and therefore, optimizes the delivery of soluble mediators and inflammatory cells to the site of infection [83]. NO is produced by the enzyme called NO synthase (NOS) [83] and the three NOS isoforms are endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). eNOS and nNOS are constitutively expressed whereas iNOS is induced in response to a variety of stimuli which include microbial products and cytokines (TNF-α, IL-1, IFN-γ) [65,83]. Monocytes/macrophages, neutrophils, endothelial cells and smooth muscle cells are all capable of producing NO by iNOS. NOS utilizes the essential amino acid L-arginine as its substrate to yield NO and L-citrulline [84]. In the absence of the substrate, NOS generates a product called superoxide. The production of NO, in combination with the vasodilator substance prostaglandins, will subsequently cause smooth muscle relaxation through a guanosine 3', 5'-cyclic monophosphate (cGMP) mechanism [65,83]. Therefore, NO that is generated in the endothelium plays an important role in regulating the vascular tone, blood pressure and blood flow distribution [84,85]. Other major roles of NO are its ability to inhibit leukocyte aggregation as well as reduce platelet aggregation.

During sepsis, LPS and pro-inflammatory cytokines such as TNF-α, IL-1β, and IFN-γ contribute to the induction of iNOS [65,83], consequently, excessive amounts of NO are produced. The role

that iNOS plays in the pathogenesis of septic shock has been the subject of controversy for many years. Evidence supporting this hypothesis is found in animal models of sepsis and septic shock which have shown increased plasma levels of NO byproducts such as nitrate and nitrite [83]. Administration of inhibitors to NO synthesis prevented LPS induced hypotension and showed decreased plasma levels of nitrate and nitrite [83]. Interestingly, administration of iNOS inhibitors several hours post-endotoxin introduction were shown to reverse the effects of septic shock which include hypotension and vascular leaks [83]. Other evidence supporting NO's role in the pathogenesis of sepsis include studies using iNOS knock-out mice. LPS and cecal ligation puncture (CLP) models of septic shock both demonstrated increased survival and clinical improvements in the iNOS knockouts when compared to wild type mice [83]. In contrast, several experimental studies have demonstrated that administration of inhibitors of NO synthesis had no beneficial effects. It has also been shown that in human models of sepsis and septic shock, measurements of plasma levels of nitrite and nitrate in patients revealed elevated levels. Treatments with certain inhibitors of NO synthesis in clinical trials have demonstrated short term hemodynamic effects which include improvements in the mean arterial pressure and systemic vascular resistance. However, a decrease in the cardiac output was also noted [83]. Recent clinical trials had to be terminated due to increased mortality in the treatment group. In summary, NO may contribute to the cardiovascular alterations observed during sepsis and septic shock including vasodilation, hyporeactivity to vasopressors and myocardial dysfunction. NO rapidly reacts with other free radicals, for example superoxide radical (O_2^-), to generate highly reactive oxidant species including peroxynitrite that lead to tissue damage. However, blocking NO synthesis as a therapeutic intervention for sepsis is still a matter of debate.

1.11.7 Cell-adhesion Molecules and Chemokines

The acute inflammatory response to infection requires the recruitment of leukocytes to the site of inflammation in order to eradicate the pathogen. Bacterial products and cytokines will induce the expression of adhesion molecules on the surface of endothelial cells and leukocytes to mediate this interaction [75,86]. Adhesion molecules play vital roles in processes which include cell growth and differentiation. However, during an inflammatory response, adhesion molecules are responsible for immune cell transmigration and response [75,86]. Endothelial cells and leukocytes possess complementary surface adhesion molecules which allows the leukocyte to roll along the endothelium following a gradient of chemokines and the leukocyte can eliminate the pathogen by phagocytosis [65,86]. Initially, leukocytes will migrate to the endothelium, also called tethering/rolling, through selectin mediated interactions. Selectins are a family of surface adhesion molecules comprised of three members. These include E-selectin, P-selectin and L-selectin [65,86]. P-selectin is constitutively expressed and stored in endothelial cells and platelets. Upon stimulation by leukotrienes, oxidants and complement, P-selectin will be expressed on the surface of endothelial cells. E-selectin is expressed by endothelial cells in response to LPS and cytokines such as IL-1. L-selectin is expressed on leukocytes and, upon endothelial cell activation, undergoes a conformational change. Once released, L-selectin will accumulate in the plasma as a soluble protein [87]. E-selectin will mediate the initial interaction with activated leukocytes. The leukocytes will tether and roll along the endothelium and subsequently become firmly adhered to the endothelium through integrin binding. Integrins are expressed on the surface of leukocytes and will interact with ligands such as intercellular adhesion molecules (ICAMs) on the surface of endothelial cells [65]. This firm adherence will allow for subsequent diapedesis and chemotaxis [75].

Once the leukocytes are firmly attached to the endothelium, they will penetrate the endothelium and migrate to the extravascular inflammatory area. This process is also mediated by adhesion molecules which include the platelet-endothelial cell adhesion molecule-1 (PECAM-1) [65,75]. Therefore, the tethering/rolling, adherence and diapedesis are mediated by interaction of adhesion molecules on leukocytes and endothelial cells which ultimately leads to the infiltration of leukocytes to the site of infection [65,75].

Adhesion molecules are an integral part of the host response through the recruitment of leukocytes to the site of infection for subsequent elimination of the pathogen. However, excessive expression and activation of these molecules will lead to tissue injury as seen during sepsis and septic shock [88]. During sepsis, E-selectins, P-selectin, ICAM-1, ICAM-2 and VCAM-1 are overexpressed and cause the recruitment of leukocytes at non-infectious locations [88]. Consequently, there is destruction of the endothelial barrier which results in loss of fluid from the circulation into the surrounding tissue. Therefore, during sepsis, the overexpression of adhesion molecules induces the destruction of healthy tissue.

Chemoattractants such as complement components and chemokines also play a pivotal role in the elimination of the invading pathogen [65]. These soluble molecules are necessary for the migration of leukocytes to the site of infection [89]. In addition, chemokines have been shown to be involved in other biological processes including cellular differentiation and activation, angiogenesis/angiostasis, lymphocyte recirculation and immune surveillance [89]. Four families of chemokines have been identified thus far based on the cysteine residues in the N-terminus [65]. These include: CCL1 through CCL28; CXCL1 through CXCL16; XCL1 through XCL2; and CX_3CL1 [89]. Chemokines are further classified as being either constitutive or inducibly expressed. Inducible chemokines play a role in the recruitment of cells to the site of injury [89].

For example, IL-8 (CXCL8), RANTES (CCL5), MIP-1β (CCL4), MCP-1 (CCL2) and IP-10 (CXCL10) are induced in response to stimuli such as LPS, IL-1 and TNF-α [89].

Chemokines have been shown to play a key role during sepsis through immuno-modulating mechanisms. A correlation between chemokines and tissue pathology, organ dysfunction and failure has been documented [89]. Elevated levels of IL-8 (CXCL8) have been measured in the blood of septic patients [89]. IL-8 plays a role in the recruitment of neutrophils. Increased levels of MCP-1 (CCL2) have correlated with the disease course of sepsis. Experimental data have revealed increased expression of MIP-1α (CCL3) and RANTES (CCL5) upon LPS stimulation [89]. Similarly, increased levels of MIP-1α (CCL3) and RANTES (CCL5) have been documented in human septic patients [89]. Therefore, chemokines are also implicated in the pathogenesis of septic shock.

1.11.8 Complement System

The complement system is also an integral part of the innate immune response. Its main role is to promote inflammation and eliminate the pathogen. The complement system is mostly involved in controlling bacterial infections, preferentially Gram-negative infections [80]. Microbes have been shown to activate the complement system during tissue injury and cellular injury associated with burns or traumas. There are three major complement pathways that ultimately lead to the destruction of the invading organism. These include: the classical pathway activated by surface bound IgM or IgG antibodies; the alternative pathway stimulated by microbial surface molecules; and the lectin pathway activated in response to mannose-binding lectin [80]. Consequently, these pathways, through a series of enzymatic cascades, will generate C3a and C3b, cleavage products from the complement component C3 [65,91]. C3a is a neutrophil

chemoattractant whereas C3b will promote phagocytosis by binding to the surface of microbes. C3b is able to initiate the cleavage of the complement component termed C5 by associating with other components to form a proteolytic complex [65]. The cleavage products of C5 are C5a and C5b [50]. Similar to C3a, C5a is a chemoattractant for neutrophils and also alters the vascular permeability at the site of infection [91]. The cleaved C5b proteins will form pores on membrane structures by associating with other factors such as C6, C7, C8 and C9 which ultimately lead to cell lysis.

Therefore, an inflammatory response will activate the complement system and produce inflammatory mediators such as C5a and C3a which can activate neutrophils and thus amplify the host response. During sepsis, the complement system is overactive and there is excessive production of C5a [91,92]. Recent evidence has shown that the amount of C5a in the serum correlates to the severity of the disease [92]. The generation of C5a compromises neutrophil function during sepsis.

1.11.9 Coagulation

Recently, the interaction of the coagulation pathway with the inflammatory cascade has been implicated in the pathogenesis of sepsis and MODS [93]. The coagulation pathway is activated in response to tissue injury or infection. There are two pathways in the coagulation cascade consisting of the intrinsic pathway and the extrinsic pathway [65]. The intrinsic pathway is generally activated in response to tissue injury whereas the extrinsic pathway is triggered by the production of tissue factor (TF) [65]. These pathways will converge and lead to the increased production of thrombin. Consequently, thrombin will cleave fibrinogen into fibrin forming a blood clot [65].

During sepsis, the extrinsic pathway is the primary mediator of the coagulation response to the infection [65]. TF is produced by endothelial cells and monocytes in response to cytokines (TNF-α, IL-1 and IL-6) and C-reactive protein [7,65,94]. In turn, this triggers the extrinsic coagulation pathway which ultimately leads to the formation of thrombin. In addition, the coagulation cascade will contribute to a pro-inflammatory response. For example, production of thrombin and tissue factor-VIIa can induce the production of pro-inflammatory cytokines such as TNF-α. Therefore, TF plays a key role in mediating the interaction between inflammation and coagulation [65].

As a protective mechanism to prevent an uncontrolled pro-coagulant state, the body produces several factors to counteract the coagulation cascade. The most common factors are anti-thrombin (AT), the protein C and protein S system and the tissue factor pathway inhibitor (TFPI) [65,94-96]. Protein C and its cofactor protein S play an important role in mediating the coagulation and inflammatory responses [65,93,94]. Protein C can inactivate factors which include Va and VIIIa and this inactivation delays the clotting cascade. Furthermore, protein C possesses anti-inflammatory properties by inhibiting the transcription factors NF-κB and AP-1 [93,96]. During sepsis, protein C, protein S and AT are being depleted by the continuous activation of the coagulation pathway and therefore are present in very low levels [65,93,96]. Increased mortality has been shown to correlate with low levels of protein C in septic patients.

In addition, during sepsis, the fibrinolytic system which is involved in mediating the removal of clots or thrombus from injured tissue once the homeostasis is restored, is inhibited [7,94,96-99]. The two main inhibitors of this system are plasminogen activator inhibitor-1 (PAI-1) and thrombin activatable fibrinolysis inhibitor (TAFI). PAI-1 levels are increased in response to LPS thereby leading to impaired fibrinolysis further promoting coagulation [94,98].

The overwhelming immune response to the infection leads to an excess production of many inflammatory and non-inflammatory mediators as described above. These mediators collectively lead to cellular dysfunction. As a result, diffuse tissue injury in both affected and previously unaffected locations will occur. The combination of the uncontrolled inflammatory and coagulation responses will amplify the host response and result in tissue hypoxia and ischemia. Consequently, there will be deterioration of organ function and associated metabolic abnormalities potentially leading to death.

1.12 Apoptosis

Apoptosis is a highly regulated physiological process that serves to eliminate unwanted cells. This process, often referred to as programmed cell death, is critical during development and is necessary in maintaining homeostasis and to clear damaged or abnormal cells [99-103]. Apoptosis can be morphologically distinguished from other types of cell death by the presence of membrane blebbing, chromatin condensation, apoptotic body formation and DNA fragmentation [99,101-103]. Apoptosis is a conserved evolutionary mechanism that utilizes proteases called caspases to initiate and execute cell death [104].

1.12.1 Caspases

Caspases play a critical role in the initiation and execution of apoptosis. Currently there are 14 mammalian caspases that have been identified [102,103,105]. Based on homology sequences, the family of caspases has been categorized into three subfamilies. These include the inflammatory mediators caspase 1, 4, 5, 11, 12, 13 and 14; the apoptosis initiators caspase 2, 8, 9 and 10; and the apoptosis executioner caspase 3, 6 and 7 [102,103,105,106]. Each member of the caspase family is constitutively expressed as proenzymes and contains 3 domains; an N-terminal

prodomain, a large subunit and a C-terminal small subunit [103,105]. Initiator caspases contain an extended N-terminal prodomain of >90 amino acids. In contrast, effector caspases have a smaller prodomain of approximately 20-30 amino acids [103]. The long prodomain in the initiator caspases contains sequence motifs that allow them to interact with adaptor molecules. For instance, caspases 8 and 10 contain two death effector domains (DED) whereas caspases 2 and 9 contain a caspase-recruitment domain (CARD) [105,107]. Activation of caspases require dimerization and/or proteolytic cleavage [108,109]. For example, caspase 9 is generally present as monomers and must cluster through adaptors such as APAF-1 which is required for activation [102,104]. Proteolytic cleavage of caspases, which is generally required for their activation, are cleaved immediately following an aspartic acid residue [102]. This residue is located between the N-terminal prodomain and the large subunit as well as in the interdomain linker region separating the large and small subunits [102]. The initiator caspases will generally act upstream of the effector caspases [102,110]. An example of a caspase activated cascade is the activation of the initiator caspase 8 which leads to the activation of the effector caspase 3. A substrate of this effector caspase is DNA fragmentation factor (DFF) 45. In the inactive form, DFF 45 is complexed to the nuclease DFF 40 thereby inhibiting its activity [102,110,111]. Upon cleavage by caspase 3, DFF 45 releases DFF 40 and consequently allows DFF 40 to cleave chromatin.

1.12.2 Inhibitors of Apoptosis

Cells are able to negatively regulate the activity of caspases through a family of proteins called inhibitors of apoptosis (IAPs) [112]. Thus far, eight mammalian IAPs have been discovered including XIAP, c-IAP1, c-IAP2, ML-IAP/Livin, ILP-2, NAIP, Bruce/Apollon, and survivin [112-114]. These proteins inhibit apoptosis through several mechanisms, for example ligands and signal transducers in the TNF-α pathway may be blocked by these molecules [102]. In

addition, pro-apoptotic members of the Bcl-2 family as well as cytochrome c may be targeted and blocked by IAPs [102]. More importantly, IAPs may directly interact with caspases 3, 7 and 9 [112,115]. For example, caspase 9 is primarily inhibited by a member of the IAP family called XIAP. Similarly, XIAP is the predominant inhibitor for caspases 3 and 7, however, other members of the IAPs such as c-IAP1 and c-IAP2 are also capable of inhibiting the caspase activity but to a lesser extent [103]. IAP family members are characterized by the presence of one to three copies of a baculoviral IAP repeat (BIR) domain [103,112]. XIAP, c-IAP1 and c-IAP2 all contain three tandem BIR copies along with a RING zinc finger domain at their C-termini [103,112]. Despite the presence of three BIR domains, data has shown that these domains are not equivalent in binding and inhibiting caspases. For example, XIAP's BIR3 domain potentially inhibits caspase 9 whereas the linker region between BIR1 and BIR2 inhibit caspase 3 and 7 [103,112,116,117]. RING domains which possess an E3 ubiquitin ligase activity have been shown to be essential in the targeted degradation by the ubiquitin-proteosome system [105,116]. Ubiquitin is covalently attached at a lysine residue of the target protein. The addition of one ubiquitin (monoubiquitination) results in the subcellular localization of the protein. The addition of oligomeric ubiquitin chains (polyubiquitination) results in protein degradation by the 26S proteosome. Ubiquitination is accomplished through a series of steps starting with ubiquitin conjugation to E1, E2 and finally being transferred to the target protein by E3 (ubiquitin ligase enzyme) [115-119]. Therefore, IAPs are able to self target for degradation [115]. For example, the C-terminal RING domain will initiate polyubiquitination at a lysine residue in the XIAP polypeptide. Therefore, XIAP's autoubiquitination would hinder its function of inhibiting apoptosis and allow cell death to occur more readily. In addition, IAPs are also able to degrade other proteins which include caspase 3 and Smac/DIABLO [115,116]. c-IAP1 and c-IAP2 also

possess a CARD domain near their RING zinc finger domain which suggests a possible CARD-CARD interaction between c-IAPs and caspases. In summary, IAPs are able to inhibit apoptosis either by binding, inhibiting or degrading caspases.

1.12.3 Intrinsic Pathway

There are two main pathways for the activation of caspases; the intrinsic pathway and the extrinsic pathway. The intrinsic pathway also known as the mitochondrial pathway is activated by intracellular stimuli such as DNA damage (p53), nutrient starvation and growth factor withdrawal [102,103,108]. The mitochondrial pathway serves as an amplification mechanism for the receptor mediated apoptosis. The key element in this pathway is the release of pro-apoptotic factors from the mitochondria which include cytochrome c and apoptosis inducing factor (AIF). When these factors are released, a cytosolic complex called the apoptosome will form in the presence of dATP which consists of cytochrome c, apoptotic protease activating factor-1 (Apaf-1) and procaspase 9 [102,104,108]. More specifically, cytochrome c will bind to the cytosolic protein Apaf-1 and cause a conformational change which leads to Apaf-1 activation. The Apaf-1/cytochrome c will oligomerize into a heptameric structure similar to a wheel in the presence of dATP. The centre of the heptameric structure contains seven N-terminal CARD domains which interacts with the CARD domains of individual procaspase 9 molecules [104]. The proximity of procaspase 9 molecules will lead to dimerization and activation of caspase 9. In turn, procaspase 3 will be recruited to the complex where caspase 9 will cleave the effector caspase into its active form. Caspase 3 will complete the execution phase of apoptosis by cleaving its substrate DFF 45 and allowing the nuclease to fragment the chromosomal DNA [102,104].

1.12.3.1 Bcl-2 Family

Important regulators of the mitochondrial pathway are the members of the Bcl-2 family which serve as gatekeepers for the mitochondria [103]. Also, they play an essential role in the crosstalk between extrinsic and intrinsic pathways to amplify the response [103,120]. To date, more than two dozen members have been categorized as belonging to the Bcl-2 family [103]. All members contain at least one of four characteristic Bcl-2 homology domains (BH1-BH4) [102,121,122]. According to their structure and function, the Bcl-2 proteins are divided into three categories: 1) pro-survival including Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, A1 (Bfl-1), NR-13 and Boo (Diva) contain all four BH domains [102,107,123]. These anti-apoptotic proteins are inhibitors of Bax/Bak. This inhibitory action is accomplished by either direct binding to Bax/Bak, prevention of Bax/Bak binding to mitochondrial proteins or by counteracting the effects of Bax/Bak on the mitochondria [107,121]; 2) pro-apoptosis including Bax, Bak and Bok (Mtd) which share sequence homology to Bcl-2 in the BH1, BH2 and BH3 domains [102,107,123]. These proteins exist as monomers but upon stimulation, will homo-oligomerize and form pores on the mitochondrial membrane thereby allowing the release of apoptotic factors such as cytochrome c [103,107,121]; 3) BH3-only family including Bid, Bim, Bad, Bik, Blk, Hrk (DP5), Bnip3, Bim_L, and Noxa contain the BH3 domain and exhibit pro-apoptotic activity [122]. BH3-only proteins will act mainly by binding and neutralizing the pro-survival proteins Bcl-2/Bcl-X_L. The inhibition of Bcl-2/Bcl-X_L will allow the activation of Bax/Bak and trigger mitochondrial permeabilization [102,121-123]. The members of the BH3-only family are activated by a variety of stresses. For example, Bid, Bad and Bim are activated by death receptors/caspase 8, glucose deprivation and cytokine withdrawal, respectively [107]. According to the rheostat model, the levels of Bcl-2/Bcl-XL versus Bax/Bak are at the core of the cell's decision to survive or die [107].

1.12.3.2 Smac/DIABLO

Smac/DIABLO (second mitochondria-derived activator of caspase/direct IAP-binding protein with low pI) is a protein that is normally localized to the mitochondria. After exposure to lethal apoptotic stimuli, Smac/DIABLO is released into the cytosol from the mitochondria with cytochrome c and mediates apoptosis [103,114]. Cytochrome c activates Apaf-1 whereas Smac/DIABLO promotes caspase activation by associating with the apoptosome and by negatively regulating the IAPs. Smac/DIABLO binds to IAPs thereby preventing the IAP-caspase interaction [103]. For example, Smac/DIABLO binds to the BIR3 domain of XIAP [105]. Since Smac/DIABLO has a dimeric scaffold, it can bind more tightly to XIAP as opposed to the monomeric caspase 9 protein [103]. Consequently, Smac/DIABLO-XIAP complex undergoes ubiquitination and proteosome degradation resulting in significant XIAP depletion and allows for caspase activation [103]. In addition, evidence has been provided that implicates Smac in TNF-α-mediated caspase 8 activation [124].

1.12.4 Extrinsic Pathway

The extrinsic pathway is activated by binding of an external stimulus such as Fas ligand (FasL) and TNF-α to its cognate cell surface receptor [107]. These death ligands are homotrimeric and thus cause oligomerization of the receptors upon ligand binding. The receptors contain a death domain (DD) in their intracellular region which will form homotypic interactions with adaptor proteins such as Fas-associated death domain (FADD) and TNFR-associated death domain (TRADD) [102,107]. Subsequently, these receptor-associated adaptor molecules will aggregate and recruit initiator caspases, such as caspase 8 or 10 through their common DED. For example, FasL binding to its receptor Fas will cause a conformational change on the cytoplasmic region of

the receptor. The DD of Fas will interact with the DD of FADD. FADD also contains a DED and thus is thought to interact with the prodomain of procaspase 8 which contains two DEDs. Consequently, procaspase 8 will oligomerize and therefore complete the formation of the multicomponent death-inducing signal complex (DISC) [107,108]. Caspase 8 is possibly activated in the DISC by an induced proximity model. This model is based on the theory that a high concentration of procaspase 8 will lead to autoproteolysis and subsequent activation of the caspase cascade [102,107,108]. TNF-α signalling involves the formation of two complexes based on a temporal manner as previously described. Complex I comprised of TNFR1, TRADD, TRAF2, RIP1, and cIAP1 will form upon TNF-α binding to its receptor. TRADD-based complex dissociation from the receptor leads to the recruitment of the FADD and procaspase 8 formation of Complex II [70]. The balance between complex I and complex II is potentially regulated by a protein called c-FLIP [70]. c-FLIP is expressed by NF-κB through the activation of complex I. c-FLIP is a caspase 8 inhibitor since it is structurally similar to caspase 8 but lacks the proteinase site [70,102,108]. Therefore, c-FLIP can be recruited to the complex but is not able to transduce the death inducing signal. Thus, complex II is able to actively participate in cell death when NF-κB activation is inadequate. Activation of caspase 8 leads to the cleavage of downstream effector caspases such as caspase 3 which ultimately leads to DFF 40 activation.

The extrinsic pathway will require the activation of the intrinsic pathway for an amplification mechanism in certain cell types. In Type I cells, the production of caspase 8 is sufficient to activate caspase 3 and the cell is able to directly undergo apoptosis. In Type II cells, the amount of FADD being recruited is very low resulting in a limited amount of active caspase 8 [139]. Therefore, the extrinsic pathway will require the involvement of the mitochondria to execute apoptosis. The crosstalk between these two pathways is dependent in part on the cleavage of the

Bcl-2 family member called Bid. Cleavage of the BH3 domain of Bid will release a proapoptotic fragment called truncated Bid (tBid) [120]. Subsequently, Bax/Bak will homo-oligomerize and allow the release of proapoptotic factors such as cytochrome c ultimately leading to the formation of the apoptosome and subsequent cell death.

1.13 Apoptosis and Sepsis

Apoptosis is a process that has been shown to be misregulated during sepsis and septic shock [140-145]. Factors including reactive oxygen species (ROS), endotoxin, Fas ligand and cytokines (TNF-α, IL-1β, IFN-γ, IL-8, IL-16 and IL-10) are important mediators of the apoptotic cascade [109,141,146]. For example, treatment of cardiac myocytes with cytokines led to apoptosis of these cells [147]. More specifically, TNF-α was added at increasing concentrations ranging from 1 to 4 nmol to adult rat cardiac myocytes. These results showed a correlation between increasing TNF-α concentration and increased apoptosis as measured by comet, tunnel and genomic DNA laddering experiments [147]. In addition, Ing *et al*. also observed that cytokines induce apoptosis. A combination of TNF-α (25 ng/mL), IL-1β (5 ng/mL) and IFN-γ (100 U/mL) were used to treat neonatal cardiac myocytes which resulted in apoptosis as determined by propidium iodide staining, genomic DNA laddering and PARP cleavage [148]. In addition, the synergistic combination of cytokines induced apoptosis in a NOS dependent fashion since this effect was reversible in the presence of the iNOS inhibitor N-methyl arginine (NMA) [148].

Evidence of apoptosis is also observed in cells of the immune system, intestine, liver and lung in experimental live-infection studies of sepsis [149-152]. Genomic DNA laddering was apparent in the liver, lung, kidney and caecum of an endotoxin shock mouse model of sepsis where LPS was injected at a concentration of 5 mg/kg [153]. A confirmatory tunnel assay was employed to

reveal LPS induced apoptosis in the liver. Hotchkiss *et al*. utilized a cecal ligation and puncture model of sepsis to report increased lymphocyte apoptosis in the thymus, spleen, ileum, colon, lung, kidney and muscle. Furthermore, Hotchkiss and colleagues showed that apoptosis in lymphoid organs is related to poor outcome in their animal model of sepsis [109,154]. Systemic administration of caspase inhibitors as well as overexpression of the Bcl-2 gene led to an increase in survival rate and decreased apoptosis in the thymus and spleen as determined by DNA laddering and tunnel assay [154]. Neviere *et al*. reported a 3-fold increase in rat myocyte apoptosis and activation of caspase 3, 8 and 9 in an endotoxic shock animal model [155]. Carlson and colleagues recently reported that LPS induces a TNF-α dependent caspase cascade to mediate cardiac apoptosis. Carlson *et al*. performed an experiment which involved the injection of 4 mg/kg of LPS to the intraperitoneum of mice and detected cardiac apoptosis contributing to the cardiac dysfunction [156]. Similarly, Lancel *et al.* reported that endotoxin induced myocardial dysfunction which utilized the death receptor pathway using a rat model of sepsis. Overexpression of Bcl-2 prior to LPS administration prevented myocardial dysfunction in rats [157]. Burn injury, similarly to septic shock, is associated with elevated levels of circulating pro-inflammatory cytokines and reversible myocardial depression [158,159]. Lightfoot *et al*. reported a marked increase in apoptotic cells in the left ventricle of the adult rat heart 24 h and 48 h after burn trauma. The increasing level of myocyte apoptosis was correlated to a decrease in cardiac function [158].

Administration of pan-caspase inhibitors has improved survival by 40-45% in experimental animal models [160]. Oberholzer *et al*. reported that local injections of a specific caspase 9 inhibitor, Z-LEHD-fmk, into thymi of septic mice led to increased survival [161]. Further, introduction of broad spectrum caspase inhibitors z-VAD.fmk and z-DEVD.cmk 2 h post

endotoxin challenge led to improved endotoxin-induced myocardial dysfunction, reduced caspase activation and reduced myocyte apoptosis [162]. In addition, intratracheal administration of short interfering RNA (siRNA) directed against Fas (Fas-siRNA) in a septic mouse model decreased pulmonary apoptosis and neutrophil infiltration [163]. *In vivo* delivery of siRNA targeted against Fas or caspase 8 in a CLP mouse model of sepsis led to an increased survival of 50% with a maintained suppressive effect for up to 10 days [160,164].

Human studies of septic shock have suggested increased apoptosis primarily in cells of the immune system although the phenomenon has also been noted in intestinal epithelial cells [145,160,166-168]. Despite the fact that overt increased myocyte apoptosis has not been reported in the heart in spontaneous disease or in live infection models of sepsis and septic shock, recent publications suggest that preapoptotic signalling may occur contributing to myocyte cellular stress and cytopathic changes in the heart leading to depressed myocardial contractility [145,157,165]. Therefore, widespread preapoptotic signalling may be implicated in the pathogenesis of septic shock by causing cellular dysfunction which progresses to organ failure including myocardial depression.

1.14 Influenza and Sepsis

Influenza A is a negative sense single stranded RNA virus that belongs to the Orthomyxoviridae Family. This enveloped virus contains 8 segments of viral RNA which encodes 11 viral proteins. Influenza A infects humans and is the causative agent of the flu. Annually it infects approximately 5 to 15% of the population worldwide and results in an estimated 250 000 to 500 000 deaths a year. The nature of influenza A replication results in a high mutation rate which results in the need for seasonal vaccinations. In addition the zoonotic nature of the influenza

virus allows for recombination of viral segments from different strains creating new variants that have not been encountered before. This type of mutation is the method by which pandemic strains of the flu arises. Infection with influenza results in a respiratory illness that for most individuals is self-limiting. However in susceptible populations which include individuals with pre-existing pulmonary or cardiac conditions, the very young and the elderly fatal complications may arise. The most serious of these is the development of viral pneumonia which may be accompanied by secondary bacterial infections. Progression of pneumonia leads to the development of acute respiratory distress syndrome (ARDS), acute lung injury (ALI) and potentially respiratory failure. This progression is a combined effect of the host immune system response to influenza infection and the viral infection itself.

1.15 Influenza A Transmission

The influenza A virus results in 3 to 5 million severe cases globally each year. In the United States the Centre for Disease Control estimated over a 30 year period from 1976-77 season to 2006-2007 season there were an average number of deaths of 23,607 per year. Interestingly the range of influenza associated deaths over this period varied from a low of 3,349 to a high of 48,614 deaths. This highlights the variable nature and pathogenicity of different influenza strains [181]. The authors of this report also noted that seasons where H3N2 was prominent, the deaths associated with influenza was 2.7 times higher than in seasons where other strains were prominent. The variability of different strains also plays a role not only in the pathogenicity of influenza but also in its ability to be transmitted from person to person [181]. The influenza A virus is responsible for seasonal epidemics and infrequently global pandemics of the flu as was the case with pandemic H1N1 in 2009. Seasonal outbreaks of the flu are a result of continual

mutation of this virus with regards to the proteins recognized by the immune system while pandemic strains result from antigenic shifts which completely change the viral proteins the immune system responds to. H1N1 is estimated to be responsible for approximately between 151,700 and 575,400 deaths globally [182]. Although the risk factors for development of severe complication from the flu were similar between seasonal and H1N1 infections for individuals with chronic lung diseases, pre-existing cardiac disease and pregnancy they differed in that obesity and people of younger ages cohorts (younger than 65) developed greater complications with H1N1 then those with seasonal strains [183].

The influenza virus is transmitted by three main modes which are 1) direct contact with an infected fomite 2) inhalation of droplets and 3) small particle aerosols. Infected droplets are spread primarily through coughing while aerosols are created during sneezing, speaking or breathing [184]. Infected aerosols with particle sizes below 5 μm have been shown to remain aerosolized for 1 hour [185]. To spread from one person to the next the influenza virus must remain viable while in the aerosol or on inanimate objects [186]. Viability of influenza outside of the body has been studied and factors affecting the viability of this virus have been identified. Relative humidity is one factor studied. The highest viability was associated with humidity levels close to 100% which mimics the levels within the respiratory system [187]. Infectious aerosols released in the air where the relative humidity ranged from 50% to 84% displayed a steady decrease in viability. When the humidity level dropped below 50% viability of the influenza virus increased [187]. The variation of viability was associated with aerosol dehydration caused by relative humidity levels which increased the salt concentration to a threshold range where the protein content of respiratory aerosols protected the virus from further decline in viability [188]. The changes in humidity are also associated with the different seasonal activities of influenza

over different regions of the globe. Temperate climate regions generally experience high influenza activity during the times of lowest humidity and coldest temperatures while tropical regions experience influenza peaks during their rainy season where the humidity levels are close to 100%. In both regions the tendency of people to congregate indoors also corresponds to this timing [189].

Infection with influenza A virus results in developing the flu. The symptoms of a typical uncomplicated influenza infection are fever, chills, rhinorrhea, nasal congestion, sinus pain, sore throat, cough, headache, anorexia and myalgia [190]. The influenza virus generally does not directly cause these symptoms but triggers an immune response that causes their development [191]. Macrophages continually monitor the epithelium of the airways. In the presence of the influenza virus macrophages become activated and trigger the acute phase response. Release of chemokines from activated immune cells further recruits more immune cells to the infected tissue resulting in inflammation that triggers systemic symptoms associated with the flu [190]. Fever is the most common symptom of the flu and is caused by the response to the release of cytokines including TNF-α, IL-6 and IL-1 [191]. These cytokines whether they are produced locally in the lung or systemically produce the systemic effect of fever. These cytokines cross the blood brain barrier reaching the central nervous system. There they interact with the vagus nerve and signal to the temperature control area of the hypothalamus. This initiates signalling pathways that results in the hypothalamus releasing mediators that cause reflex shivering, peripheral blood vessel constriction that results in the sensation of chills. The sensation of sore throat is attributed to the production of bradykinins and prostaglandins in the airways [190].

1.16 Influenza A Viral Structure

The influenza A virus is a negative sense segmented RNA virus that contains a lipid envelope. The core of the virus particle contains 8 segments of viral RNA that are coated by a viral protein nucleoprotein (NP) [192,193]. The protein coated segments are also bound to the three protein subunits polymerase basic 1, polymerase basic 2 and polymerase acidic (PB1, PB2, PA) that comprise the viral RNA dependent RNA polymerase which is responsible for both transcription and replication of the viral genome [1194]. Together these are the components of the viral ribonucleoproteins (vRNP) which are surrounded by a protein layer made from the viral protein matrix protein 1 (M1) [195]. This protein shell is covered by the envelope which is a lipid bilayer derived from the host cell membrane. The envelope contains three viral proteins hemagglutinin (HA), neuraminidase (NA) and matrix protein 2 (M2). The lipids of the envelope are enriched with sphingolipids and cholesterol forming rafts [186]. These raft domains associate with the embedded envelope proteins HA, NA and M2. The stability of the virus particle is primarily governed by the lipids of the membrane. The envelope proteins HA and NA have a low mobility with in the envelope and reinforce the virus structure through their association with the underlying M1 protein. M1 links the vRNPs to the envelope glycoproteins contributing to an overall stabilizing effect of the virus structure [187].

The virus gains access to the body through the nose, mouth or eyes. Influenza A virus initiates an infection when it comes in contact with the mucosal surfaces of the respiratory tract. Influenza A must come in direct contact with the epithelial cells of the respiratory tract to initiate an infection. Here the virus encounters its first barrier, the layer of mucus that covers and protects the epithelial cells of the respiratory tract. The envelope glycoprotein NA cleaves sialic acid

from mucin that lines the epithelial cells. The virus attaches to the exposed cells by the second envelope glycoprotein HA. The HA protein forms a homotrimer in the envelope. The monomer of HA is composed of two polypeptides termed Ha_1 and Ha_2. Ha_1 possess the receptor binding domain while Ha_2 contains the membrane fusion peptide [198]. This protein attaches to sialic acid linked glycoproteins on the surface of epithelial cells. The type of sialic acid linkage to the glycoprotein determines the tropism of the different types of influenza A viruses towards different hosts [199]. Human influenza A viruses contains a HA which has a binding preference for an α-2,6 linked sialic acid to galactose whereas the avian origin influenza HA has a binding preference for an α-2,3 linked sialic acid to galactose. The distribution of these glycosidic residues within a species also determines the tissue tropism of influenza. The human respiratory tract contains predominantly α-2,6 linkages expressed on the surface of the epithelium. HA also is a major determinant for tissue tropism of influenza [184]. HA must be cleaved in order for it to be functional. Proteases found ubiquitously expressed in the lungs are able to cleave HA in to a functional viral protein. Strains of influenza possessing HA that can be cleaved by other classes of proteases are able to disseminate, infecting a larger range of tissues and tend to be more pathogenic [199]. In addition to sialic acid binding recent studies have identified alternative modes of influenza A entry into cells that are dependent on c type lectins and independent of sialic acid binding [200]. Other requirements for influenza A viral attachment are also being elucidated. Fibronectin an extracellular matrix protein has been identified as a requirement for certain strains of H1 and H3 influenza viruses. Binding of the virus to the cell surface induces uptake through endocytosis. This uptake has been shown to utilize clatherin dependent and caveolin dependent mechanisms [201]. Once the virus is endocytosed it remains in the maturing endosome. Influenza escapes the endosome in a pH dependent manner [202]. As the endosome

matures it acidifies which activates the M2 envelope protein. The M2 protein is a tetramer integral protein that forms a proton selective ion channel. This protein contains three major domains, a short ectodomain, a transmembrane domain that forms the ion channel and a long cytoplasmic domain [203]. Activation of M2 allows protons to enter the viral particle. At a critical pH the M1 protein that stabilizes and mediates attachment of the vRNP to the envelope proteins dissociates and undergoes a morphological change freeing the vRNP from the envelope. In addition the Ha_2 peptide of the HA protein activates allowing for the fusion between the membrane of the endosome and the viral envelope. This fusion allows for the cytosolic release of the vRNP from the endosome [204]. Once inside the cytoplasm the vRNP must enter the nucleus for transcription of viral genes and replication of the viral genome [205]. Entry of the infecting vRNP is mediated by NP which possesses a nuclear localization sequence that binds with karyopherin which is an endogenous heterodimer of karyopherin α1 with karyopherin β or karyopherin α2 and karyopherin β. Once bound by karyopherin the vRNP is ferried to and docked at the nuclear pore complex. Entry in to the nucleus is Ran 10 and p10 dependent [206]. Once in the nucleus transcription of the viral genes occurs. The mechanism of viral transcription and genome replication has been well studied but the regulation and switching from transcription to genome replication remains to be fully elucidated. Progress has been made with regards to identifying viral elements involved in regulating these processes. Umbach et al. have found that influenza A virus produces short RNA nucleotides ranging from 18 to 27 nucleotides in length. These viral leader RNAs were found to originate from each of the 8 segments 5' ends [207]. Perez et al. found that these virally encoded RNAs acted as enhancers for viral replication but they did not function as a primer for replication. These short RNAs were retained in the nucleus throughout the infection and it is postulated that they act to not only stabilize viral RNA genome

replication but to also play a role in maintaining the proper stoichiometry of each viral segment [208]. Viral proteins also have been shown to regulate viral transcription and genome replication. Widjaja et al. produced a model whereby transcription of viral genes is accomplished by the associated RNA dependent RNA polymerase. Production and nuclear accumulation of new polymerase allows for complementary RNA to be made. This serves as a template for viral genome replication which is stabilized by both the newly synthesized polymerase and nucleoprotein [209]. They also identified elements of the 3' and 5' UTR of the viral segments involved in competition for viral proteins required for replication and also demonstrated that shorter viral segments can out compete larger segments for viral proteins [209]. The viral genomic RNA is associated with viral nucleoprotein and the three polymerase subunits before being exported out of the nucleus. This requires the re-import of translated nucleoprotein and the polymerase subunits which possess multiple elements that allow for shuttling between the nucleus and cytoplasm [210]. Together these proteins form the vRNP. The vRNP forms a complex in the nucleus between the M1 protein and the nuclear export protein (NEP) formerly known as NS2 [211]. Both M1 and NEP have been shown to be required for export of the vRNP out of the nucleus into the cytoplasm which is chromosome region maintenance I (CRM1) dependent [212]. The vRNPs accumulate at the microtubule organizing centre upon exiting the nucleus. In addition to these viral proteins, a host cell protein Y-box binding protein (YB-1) has been identified as associating with this complex in the nucleus. This protein is believed to act as an adaptor for the vRNP allowing it to associate with microtubules and to target it to the rab11a recycling endosome which delivers the vRNP to the site of virus assembly [212].

Release of the virus from the cells requires assembly of the viral proteins and all 8 vRNP segments into the viral particle. This assembly occurs at the cell's plasma membrane. The viral

proteins HA, NA and M2 are transmembrane proteins that are inserted into the host's plasma membrane [213]. HA and NA are localized to lipid rafts rich in cholesterol and sphingolipids [214]. The M1 protein forms associations with the cytoplasmic portions of these transmembrane proteins. It has been demonstrated that viral assembly is selective for each of the 8 vRNPs and virions have one copy of each segment in them [213]. Initiation of the budding process involves the HA protein which appears to be necessary for efficient budding but is not absolutely required. It has been shown that HA assists in creating membrane curvature to start the bud but requires the recruitment of other viral proteins for the budding process to complete. The M2 protein plays a role in the scission of the bud from the cell. It has been shown that M2 accumulates in the neck of the bud where scission occurs. Scission of the bud occurs independently of the host endosomal sorting complex required for transport (ESCRT) [215]. Final release of the virus particles requires the enzymatic action of NA which cleaves the sialic acid moiety from the budding virus freeing it from the cell.

1.17 Viral Pneumonia

One of the most serious complications of influenza A infections is the development of pneumonia. Viral induced pneumonia is a result of a combination of extensive viral replication in the lung, particularly the lower regions and the host immune response to the virus [216]. The general progression to viral pneumonia development starts with viral replication in the lung epithelial cells and alveolar epithelium. Detection of viral infection in the epithelial cells induces the production and release of pro-inflammatory cytokines and chemokines [217]. The host response to the release of these inflammatory mediators is the recruitment and activation of leukocytes from the circulatory system into the lung. In combination with resident alveolar

macrophages the infiltration of mononuclear cells can lead to the overproduction of pro-inflammatory cytokines [218]. Cell damage results from both the replicating virus which induces both necrosis and apoptosis in lung epithelial cells and macrophages and the products from activated leukocytes including reactive oxygen species [219]. When the cell damage becomes extensive this leads to alveolar flooding where protein rich fluid accumulates in the airspace due to impaired fluid uptake from the alveolar epithelium [220]. This edema is further exacerbated by an increased permeability of the lung capillaries in response to the infiltrating leukocytes. Damage to the blood air barrier results in decreased gas exchange which results in systemic hypoxia. Autopsy examination of lung tissue from influenza induced pneumonia shows signs of edema, hemorrhage, diffuse alveolar damage and the production of hyaline membrane [183]. Acute respiratory distress syndrome (ARDS) leading to respiratory failure is the most common lethal development of influenza induced pneumonia. This syndrome is divided into three phases, the exudative, inflammatory and fibroproliferative phase [221]. Damage to sufficient numbers of type II pneumocytes from influenza infection results in a decrease of surfactant production in the lung. Lung surfactant has been shown to have immunomodulating effects on immune cells [222] . It has been demonstrated that during ARDS decreased lung surfactant results in increased PI3 kinase activity in immune cells [223]. On lung epithelial cells and alveolar macrophages the decreased surfactant production results in increased AKT activation. For neutrophil dependent damage to the lung AKT activation has been shown to be involved [224]. The ensuing alveolar flooding is a result of the impaired fluid removal across the lung epithelium due to impaired sodium transport across alveolar epithelial cells [221]. In addition the increased cytokine and chemokine production of the alveolar epithelium increases the permeability of the lung endothelium allowing for increased fluid to enter the alveolar space [224]. A further

complicating factor with influenza induced pneumonia is the development of a secondary bacterial infection in the lungs [225]. Up to half of pandemic influenza induced pneumonia was associated with a secondary bacterial infection [226]. There is also an association with the type of bacteria found coinfecting with influenza A. Most commonly associated bacterial species which have been found to cause secondary infections to influenza are *Streptococcus pneumoniae*, *Haemophilus influenza* and *Staphylococcus aureus* [227]. For these species of bacteria there is an apparent synergism with regards to virulence and pathogenicity. Coinfection with influenza or preinfection with influenza virus results in changes to the functioning of immune cells particularly neutrophils and alveolar macrophages [228]. There is also an observed increase in bacterial adherence to the lung epithelium and a concurrent immunosuppression attributed to influenza infection [229]. It has been observed in mice that influenza infection results in higher cytokine production of TNFα, IL-1β, IL-6, MIP1α and KC relative to a sole bacterial infection [230]. Although influenza A infections predisposes the host to secondary bacterial pneumonia, a recent study has demonstrated that a previous infection with influenza affords some protection against subsequent bacterial pneumonia infection with a different influenza type. This protection was attributed to non-neutralizing antibody production from the initial infection against conserved internal viral protein NP [230].

1.18 Influenza A Induced Pro-inflammatory Cellular Signalling

Dendritic cells and macrophages, which reside in close proximity to the lung epithelium produce a significant amount of TNF–α and type I IFN in response to influenza virus infection [231]. These cytokines activate intracellular signal transduction pathways in the lung epithelial cells. The highly pathogenic 1918 Influenza virus induced gene expression profiles in mouse and non-

human primate lungs which consisted of suppression of type 1 interferon antiviral response and enhanced expression of TNF- α, IFN-γ and IL-6 pro-inflammatory response. There is a dramatic increase in chemokines and inflammatory influx consisting of neutrophils, macrophages and T lymphocytes resulting in enhanced lung injury by a recombinant virus containing 1918 influenza genes HA and NA [232,233]. Interferons activate intracellular Jak-Stat pathway as well as alternative signal transduction pathways in parallel [234,235], TNF- α activates a variety of protein kinases including c-Jun N-terminal kinase (JNK), extracellular-signal regulated kinases (ERK1/2), p38 and I-kappa B kinases (IKK). TNF- α activates nuclear factor kappaB (NF-κB) by means of a kinase relay module, involving the IκB kinase (IKK) α, β, γ signalosome [236]. In addition to NF-κB, TNF- α also activates additional transcription factors such as AP1 (Fos-Jun) via JNK and activation transcription factor–2 (ATF-2) via JNK, ERK and p38 MAP kinase pathways [237-239]. Involvement of ERK, JNK, p38 MAP kinases and activation of transcription factors AP1, NF-κB and ATF-2 has been described in response to influenza virus infection [240-244]. Thus, multiple MAP kinase pathways are activated by cytokines as well as virus replication. Influenza virus propagation was impaired by inhibition of Raf/Mek/ERK pathway [240]. Furthermore, lung-specific expression of active Raf kinase resulted in increased mortality of influenza virus infected mice [241]. Targeting of NF-κB pathway for antiviral therapy in influenza infection has been shown to reduce viral titers and cytokine expression [245]. Further understanding of intracellular signal transduction pathways may lead to the development of specific inhibitors of influenza viral replication as well as inflammatory host response that are involved in lung injury.

1.19 Intracellular Detection of Influenza A Virus.

Cells of the lung epithelium have evolved receptors to detect the presence of viral nucleic acid and initiate a signalling cascade to induce an antiviral pro-inflammatory state (Figure 3). These receptors belong to a family of pattern recognition receptors each of which have a class of molecular structures which binds and activates them [246]. Influenza A virus has been demonstrated to activate toll-like receptors (TLRs), NLRP3 inflammasome which belongs to the NOD-like receptors, RIG-I like receptors (RLR), PKR and 2'-5'-oligoadenylate synthetase [247]. Activation of these receptors is the primary signal for production of pro-inflammatory cytokines and chemokines which are released to recruit and activate leukocytes from the circulation to the site of influenza infection [248,249]. The RLRs includes three members, retinoic acid inducible gene one (RIG-I), melanoma differentiation associated gene 5 (MDA5) and laboratory of general physiology 2 (LGP2). For cytosolic detection of influenza RIG-I is the primary receptor involved [250]. The vRNP and replicative intermediates contain 5'triphosphates and double stranded RNA which are the unique viral molecular patterns that this receptor binds to distinguishing foreign viral RNA from self RNA [251]. Activation of RIG-I results in a cascade of signaling events that pivots around the association with the mitochondrial outer membrane embedded protein IPS-1 [250]. Subsequently the downstream signalling leads to the activation of transcription factors NFκB and interferon regulatory factors 3 and 7 (IRF). NFκB activation is responsible for the induction of transcription for pro-inflammatory cytokines while IRF3 and 7 induce the transcription of interferon beta gene which leads to the induction of antiviral genes to create an antiviral state [252,253]. Similar to RIG-I signalling activation of TLR3 which is a receptor that resides in the endosomal membrane results in the activation of NFκB and the production of pro-inflammatory cytokines [254]. TLR3 binds double stranded

RNA. Unlike RIG-I in pulmonary epithelial cells this receptor does not contribute significantly to the production of interferon however its signalling significantly induces a pro-inflammatory state [255]. In a mouse model of influenza induced pneumonia tlr3 negative mice were found to have a protective effect with reduced mortality compared to wild type mice with increased survival being attributed to a decreased production of pro-inflammatory cytokines [254]. PKR is a 68 kDa latent protein expressed by most cells. It is induced by interferons and is activated by the binding of double stranded RNA or double stranded structures within ssRNA [256]. PKR possess two double stranded RNA binding domains which binds its RNA ligand. Binding of RNA induces a conformational change that allows for monomeric PKR to form dimers which allows for autophosphorylation and activation of its kinase domain [257]. Once activated PKR will phosphorylate its substrate eIF2α initiating a stress response in the cell [258]. This stress response is a rapid reversible inhibition of protein translation. This reduction in translation leads to a reduced translation of all mRNA. The accumulation of mRNA with in the cytoplasm triggers the recruitment of three proteins; the cytotoxic granule associated RNA binding protein (TIA-1), TIA-1 cytotoxic granule associated RNA binding protein like 1 (TIAR) and GapSH3 domain binding protein (G3BP) [259]. As these proteins bind the mRNA they trigger aggregation of these complexes together forming stress granules. PKR triggered inhibition of translation leads to the formation of these antiviral stress granules. Viral induced stress granules require PKR activation. Influenza induced stress granules recruit PKR, RIG-I, and oligoadenylate synthetase (OAS) [260]. This platform provides an efficient mechanism for interferon induction in response to viral infection. It has also been shown that vRNA colocalizes with these granules which activates RIG-I. Once activated the antiviral stress granule is recruited to the mitochondria to associate with IPS-1. The viral NS1 protein has been shown to antagonize interferon induction at

many levels. This viral protein inhibits activation of PKR, OAS, and RIG-I [261]. Through inhibition of PKR activation influenza A virus prevents antiviral stress granule formation and production of interferons while the proinflammatory arm of signalling remains intact. The OAS/RNAse L system is part of the innate immune response. OAS is a pattern recognition receptor that is activated by viral double stranded RNA [262,263]. Activation of OAS leads to the production of a unique molecule 2',5' oligoadenylate. This molecule specifically activates RNAse L [264]. Typically OAS produces trimers and tetramers of 2',5'-oligoadenlyate. Activation of RNase L requires the formation of dimers in response to binding of 2',5'-oligoadenlyate. The active dimer cleaves single stranded RNA at the dinucleotide motif of uracil adenosine or uracil uracil [265]. The resulting cleavage product possesses a 3' phosphate which acts as a substrate for RIG-I thus amplifying the signalling to interferon production [247,266]. In addition the degradation of viral RNA coupled with the inhibition of translation is the main mechanism of RNase L antiviral activity. However RNase L is not specific to viral RNA degradation and it degrades host rRNA and mRNA and can lead to apoptosis. The NS1 protein of Influenza A has evolved to inhibit the action of the OAS/RNase L system [261]. NS1 has been shown to sequester viral double stranded RNA so that it prevents activation of OAS and as a result its effector protein RNase L [267].

1.20 Influenza A and Apoptosis

The NS1 protein also inhibits the activation of caspase 3 and 7. This is accomplished by interaction of the p85 subunit of PI3 kinase with NS1. The inhibition of apoptosis by NS1 primarily through the antagonism of the production of type I interferons is necessary to allow sufficient time for the virus to transcribe all necessary viral proteins and to replicate its RNA

genome. However later in the virus life cycle apoptosis has been shown to be necessary for efficient virion production. Influenza A possesses apoptosis inducing properties [268]. The viral protein PB1-F2 interacts with two mitochondrial proteins Adenosine nucleotide translocase3 and voltage dependent anion channel 1. This interaction leads to a disruption in the outer mitochondrial membrane allowing for the release of proapoptotic mediators [268,269]. The viral infection of lung epithelial cells also results in the secretion of death ligands FasL and TRAIL which induces apoptosis through death receptors on the surface of lung epithelial cells.

The importance of the apoptotic process for efficient influenza A replication has been demonstrated in the overexpression of anti-apoptotic protein bcl-2 in epithelial cells and infecting them with Influenza A. These cells produced significantly fewer virus particles which contained misglycosylation of the hemagglutinin membrane protein. A similar phenomenon is demonstrated when caspase activation in particular caspase 3 is blocked in vitro. When caspase 3 is inhibited or the nuclear export machinery is inhibited both cause viral RNPs to accumulate in the nucleus and a large decrease in the numbers of progeny virus being formed [268].

The immune system utilizes apoptosis to eliminate influenza A infected lung epithelial cells. This is accomplished by the death ligands FasL and TRAIL. FasL is produced primarily by lymphoid cells and neutrophils. TRAIL is expressed by a wide population of cells including immune cells and epithelial cells. It has been demonstrated that recruited macrophages expressing CCR2 induces apoptosis in lung epithelial cells by means of TRAIL however not only infected epithelial cells but cells expressing low levels of death receptor 5 also undergo apoptosis which contributes to lung damage induced by the immune system [268].

Sepsis is characterized as an overwhelming pro-inflammatory systemic response to an infection. Invading pathogens will trigger a host defense response involving the excessive production of inflammatory mediators including cytokines (TNF-α, IL-1β, IFN-γ) from monocytes/macrophages and other cells. Consequently, these cytokines will amplify the host response by stimulating the release of downstream inflammatory mediators. Collectively, these mediators produce the pathophysiological abnormalities observed during sepsis and septic shock. The pathogenesis of sepsis is very complex and involves overlapping and redundant intracellular signalling pathways leading to the misregulation of processes including cell adhesion molecule regulation, iNOS induction and apoptosis. These processes are broadly regulated by STAT, IRF and NF-κB transcription factors.

1.21 Thesis Objectives

This first objective of this study was to investigate the cytokine production and cell death seen in lungs of individuals infected with pandemic H1N1 2009 influenza A. The second objective is to characterize the levels of these cytokines from the sera of H1N1 infected individuals and investigate the apoptotic inducing ability of these sera on cells in culture. The final objective is to characterize the pro-inflammatory mediators found in the sera from individuals with bacterial induced sepsis.

Chapter 2: Pandemic H1N1 influenza A

2.1 Introduction

In 2009 a novel influenza virus began to circulate through the global population. The components of this strain were identified as containing genetic segments from a swine triple reassortment strain, which contains avian and human influenza virus components, and genetic segments from Eurasian swine influenza A [270]. This strain was identified in 2 cases from California from people who did not have contact with pigs indicating this strain was able to spread from human to human. By June 2009 the W.H.O. declared a pandemic for this strain of influenza A. Like the seasonal strains of influenza A pandemic H1N1 2009 the most common clinical manifestations experienced were fever, headache, sore throat, chills and muscle aches [271]. Those most at risk of developing complications were people with pre-existing respiratory illnesses, heart conditions, pregnant women, obese people and diabetics. Similar to pandemic strains in the past severe and fatal cases of the flu were being experienced by a younger age group then from seasonal strains [271]. Since the first cases of pandemic influenza H1N1 (2009) were noted on the east coast of Canada on April 23, 2009, several thousand patients have been hospitalized with a significant subset requiring ICU care [272,273]. The largest initial outbreak of severe disease in Canada was seen during May and June in Manitoba where over 50 patients required ICU care over an 8 week period [274]. During this outbreak, seven adult deaths due to laboratory confirmed H1N1 occurred. The lung pathology of 7 of these cases is reported along with assessments of cell death and cytokine production from these samples.

2.2 Methods

2.2.1 Tissue samples and processing. All cases were confirmed by real-time reverse-transcriptase–polymerase-chain reaction assay (PCR). Complete autopsies were performed under the authority of the Provincial Medical Examiner, using standard precautions, and with the use of N95 masks. Tissue sampled for histological evaluation was fixed in 10% formalin, with routine processing to hematoxylin and eosin stained slides. Slides from four to six areas of the lung were examined in each case. Fresh tissue was submitted for viral studies and microbiological cultures. Portions of lung were collected in 10% buffered formalin and processed in a routine manner.

2.2.2 Tissue staining. For immunohistochemistry, paraffin embedded tissue sections were quenched for 10 minutes in aqueous 3% H_2O_2 then pretreated with proteinase K for 15 minutes. The primary antibody was a mouse monoclonal antibody specific for influenza A nucleoprotein (NP) (F26NP9, produced in-house) and was used at a 1:10,000 dilution for one hour. They were then visualized using a horse radish peroxidase labeled polymer, Envision® + system (anti-mouse) (Dako, CA, USA), reacted with the chromogen diaminobenzidine (DAB). The sections were then counter stained with Gill's hematoxylin.

5μm formalin fixed paraffin embedded sections of lung tissue were used for cytokine staining. Primary mouse monoclonal antibodies for TNF-α, IL-1β and IFN-γ (clones 28401, 8516 and 25718) were purchased from R&D systems. Tissues were sectioned on an American Optics Spencer 820 rotary microtome. Sections were floated in a 45˚C water bath mounted on Superfrost® Excell™ microscope slides (Fisher Scientific) and air dried. Dried slides were placed in a 45˚C incubator overnight removed and stored at room temperature until use.

Slides were dewaxed in 2 changes of xylene 5 minutes each. Washed 2 times each in an alcohol series ranging from 100% to 70% 3 minutes each and finally washed in distilled water for 5 minutes.

Antigen unmasking was performed using Vector Labs high pH unmasking solution. Slides were immersed in the unmasking solution and autoclaved for 15 minutes at 121˚C. After depressurization slides were cooled to room temperature and then washed in distilled water for 5 minutes. Slides were then transferred to a solution of tris buffered saline pH 7.4 (TBS) for 5 minutes. Next slides were blocked with a solution containing 5% horse serum (Vector Labs), 1% bovine serum albumin fraction V (Fisher Scientific), 0.3% triton X 100 in TBS (TBST) for 1 hour at room temperature. To block tissue binding of avidin and biotin slides were blocked in solutions of avidin and biotin (Vector Labs) for 30 minutes each. Next slides were incubated with primary antibody diluted 1 in 500 with 2% horse serum, 0.3% tritonX100 in TBS overnight at 4˚C. Slides were then rinsed with TBST and then washed in TBST for 5 minutes 3 times. Slides were then incubated with secondary antibody (Vector Universal Elite ABC Kit) diluted 1 in 300 with same diluent as the primary antibody for 1 hour at room temperature. Slides were rinsed for 5 minutes 3 times in TBST. Slides were quenched of endogenous peroxidase activity by incubating in a solution containing 1% hydrogen peroxide, 0.5% sodium azide in TBS for 30 minutes. The avidin biotin complex was formed by adding equal volume of each to tube (Vector Universal Elite ABC kit) and incubated for 30 minutes. After quenching the slides were washed 3 times in TBST and then incubated with the avidin biotin complex 30 minutes at room temperature. Slides were rinsed with TBST 5 minutes 3 times. Slides were developed using ImmPact™ DAB Peroxidase Substrate (Vector Labs) according to manufacturer's instructions for 10 minutes. Slides were then rinsed with water for 5 minutes and then counterstained with

Hematoxylin nuclear counter stain (Gill's Formation) (Vector Labs). Finally slides were dehydrated in an alcohol series, clarified in 2 changes of xylene and mounted with Permount™ (Fisher Scientific). After curing slides were documented with a Ziess Axiovert 200M inverted microscope.

2.2.3 TUNEL Assay. TUNEL assay was performed on 5µm sections of paraffin embedded tissues. The TUNEL assay was performed according to manufacture's protocol (Trevigen, Gaithersburg MD) with the following modifications. The sections were permeabilized with a 1:50 dilution of proteinase K in water and incubated for 20 min. at 37˚C. Manganese was used as the cation for the labeling reaction. The conjugate streptavidin HRP was incubated for 20 min. at 37˚C and the substrate TACs blue was developed for 2.5 min. Sections were counterstained in nuclear fast red, mounted with Permount (Fisher Scientific) and coverslipped. The slides were documented on an Axiovert 200M inverted microscope (Ziess).

2.3 Results

The patients ranged in age from 17 to 55 years. Five were of aboriginal origin, and two were Caucasian. There were 6 females and one male. All patients presented with a history of recent onset cough with fever and systemic symptoms of flu like illness, and were clinically suspected to have influenza. Two deaths occurred late in the third trimester of pregnancy, both cases requiring delivery by urgent caesarean section resulting in one still birth, and one live birth with a surviving infant. Only one of the deaths occurred in an individual with no known underlying medical condition. None of the individuals had received the H1N1 Influenza vaccine, with the exception of one (case 1), who received the vaccine four days prior to becoming symptomatic,

and died the following day. Table 2-1 summarizes the patient characteristics, time from presentation to death, and histopathology of the lungs at autopsy.

At autopsy gross examination revealed the lungs to be diffusely congested, consolidated and variably hemorrhagic in all cases. Correspondingly, the lungs were generally heavy, with weights ranging from 335 g to 1245 g (average 1027 g) for the right lung; and 330 g to 1340 g (average 1000 g) for the left lung (normal lung weight about 450 g). In all cases, bilateral pleural effusion which was frequently blood tinged was present, with the exception of one case in which chest tubes were in place.

The shortest duration between presentation and death occurred in cases 1 and 2, and in both cases the lungs demonstrated a similar microscopic picture. The predominant finding was striking and widespread dilatation of pulmonary alveolar capillaries and marked congestion of pulmonary vasculature in general. There was no evidence of capillaritis or histological alveolar wall damage. There was moderate pulmonary edema in one case, and evidence of edema was present within lobular septae, but inconspicuous within alveolar spaces in the other. Patchy interstitial lymphocytic infiltrates were present within the alveolated parenchyma but were generally inconspicuous. Mild peribronchiolar lymphocytic infiltrates were seen in case 1. Rare perivascular infiltrates with foci of subtle endothelialitis were seen in case 2.

Table2-2 summarizes the staining for influenza A nucleoprotein and cytokines for these 7 cases. See appendix table A-1 and figures A-1 to A-3 for additional samples. The viral protein was detected in both the nucleus and cytoplasm of infected cells (Figure 2-1). Cytokine staining was similar for all three targets TNF-α, IFN-γ and IL-1β. Weak staining was observed primarily in

the infiltrating leukocytes with no staining observed in the epithelial cells (Figure2-2, 2-3 and 2-4).

Table 2-1 Demographic and pathological findings from pandemic H1N1 2009 cases

Case	Age (years)	Sex	Co-morbidities / Pertinent Medical History	Days from onset of illness to death	Primary Lung Findings at Autopsy
1	35	F	Down Syndrome	1	Marked pulmonary capillary and vascular congestion, alveolar hemorrhage
2	46	M	None	4	Marked pulmonary capillary and vascular congestion, alveolar hemorrhage
3	45	F	Obesity, hypertension, asthma	9	Marked pulmonary capillary and vascular congestion, alveolar hemorrhage, mild diffuse interstitial pneumonitis (lymphocytic alveolitis), foci of early acute lung injury with occasional hyaline membranes

4	55	F	Obesity (BM1 47.4) Rheumatoid Arthritis, recent course of Prednisone	11	Organizing diffuse alveolar damage, with striking type two pneumocyte hyperplasia, and occasional residual hyaline membranes and airspace fibrin
5	17	F	Pregnant (third trimester)	19	Pulmonary vascular congestion, mild interstitial pneumonitis, airspace macrophages, patchy type two pneumocyte hyperplasia and airspace fibrin deposition.
6	24	F	Pregnant (third trimester)	21	Organizing hemorrhagic diffuse alveolar damage
7	32	F	Rheumatoid arthritis, Diabetes Mellitus, underlying lung fibrosis	53	Organizing acute lung injury with extensive airspace fibroplasia, vascular congestion and patchy alveolar hemorrhage, mild patchy interstitial pneumonitis.

Demographic and pathological data from 7 fatal cases of pandemic H1N1 infections

Table 2-2. Summary of immunohistochemical staining for H1N1 viral proteins and cytokines in lung tissue.

Case No.	IHC Score for virus	Location of staining	Cell types with positive staining for virus	TNF-α	IFN-γ	IL-1β
1	++++	B/A	Airway Epithelium, Type II pneumocytes, macrophages	++	+	Negative
2	+++	B/A	Type I and II pneumocytes	+	Negative	Negative
3	+	B	Airway Epithelium	+	Negative	Negative
4	+	N/A	Type II pneumocytes	Negative	Negative	Negative
5	+	C/I	Macrophages and Neutrophils	++	Negative	+
6	+	C/I*	Macrophages and Neutrophils	++	+	+
7	++	B/A	Type II pneumocytes and Macrophages	++	++	+

Immunohistochemistry staining for viral nucleoprotein and cytokines TNFα, IL-1β and IFN-γ were performed on 5μm sections of lung tissue from autopsy samples. Proteins were detected by diaminobenzidine and counterstained with hematoxylin. The level of staining is indicated by the symbol + weak staining, ++ strong staining and +++ for heavy staining. Location of staining for viral protein A= alveolar, I= interstitial, C= cytoplasmic, N= nuclear and B= both

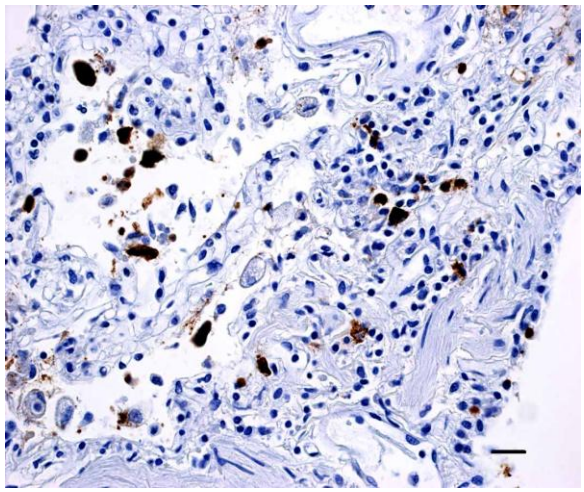
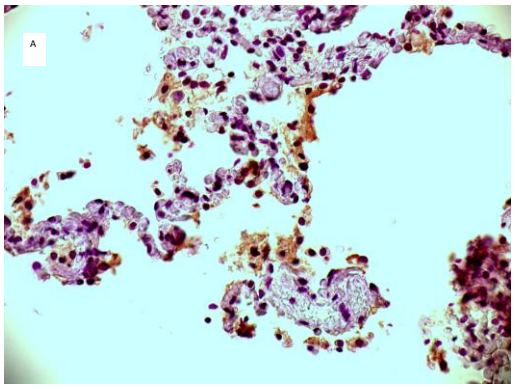


Figure 2-1 31 year old male with 1 day of severe respiratory symptoms with PCR confirmed H1N1 infection. Section of lung with immunohistochemical staining for influenza A nucleoprotein using mouse monoclonal antibody. Note heavy staining of antigen in nucleus and cytoplasm of Type II pneumocytes.

Bar = 20 µm

A



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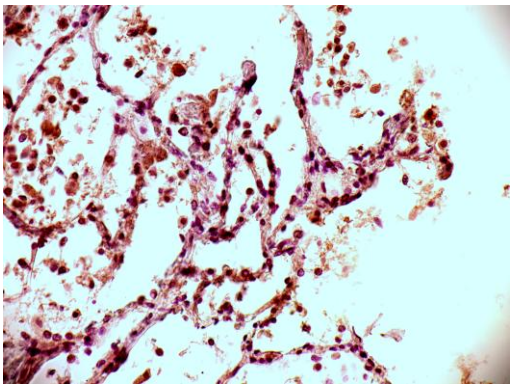

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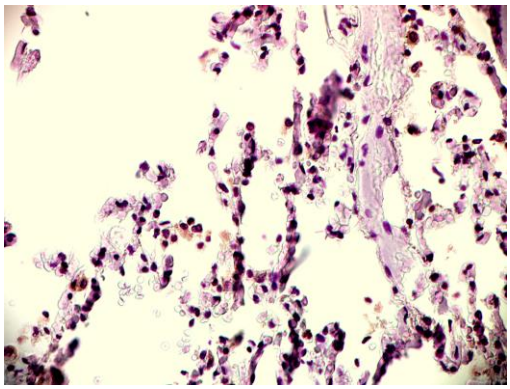

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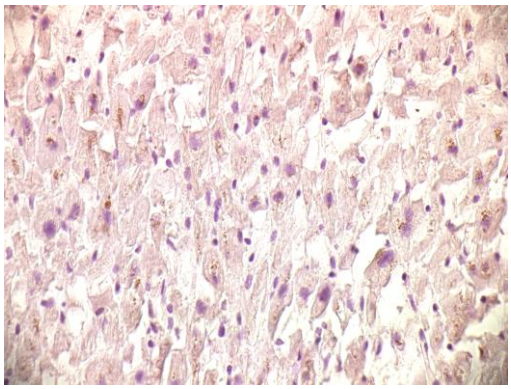

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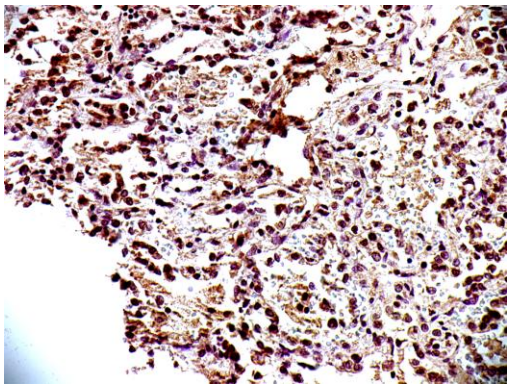

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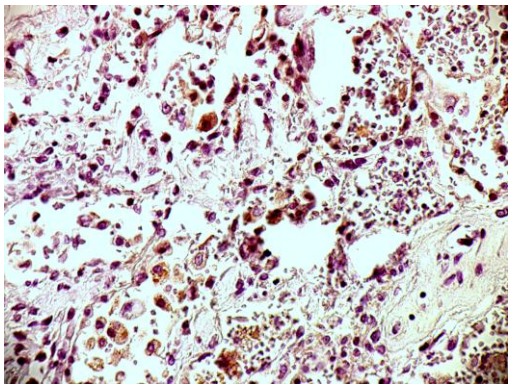

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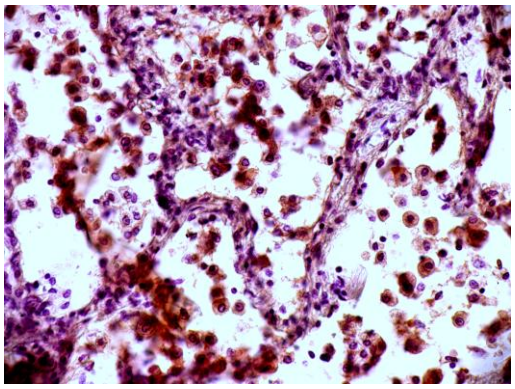
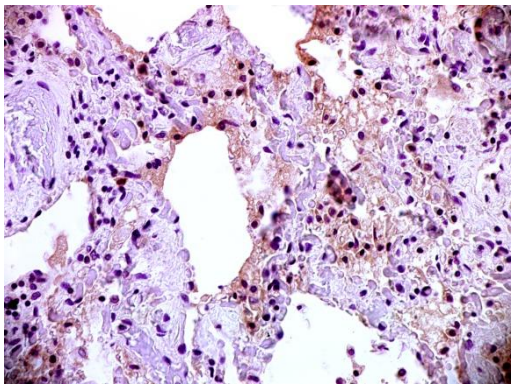


Figure 2-2. Immunohistochemical staining for TNF-α of autopsy lung tissue from H1N1 infections. 5μm sections of lung tissue where stained for TNF-α using the chromogen diaminobenzedine (DAB) (brown) and counterstained with hematoxylin (blue).

A



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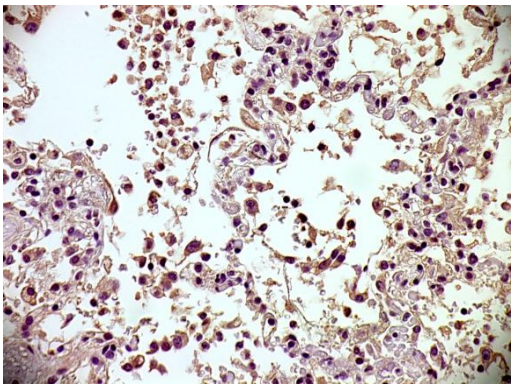

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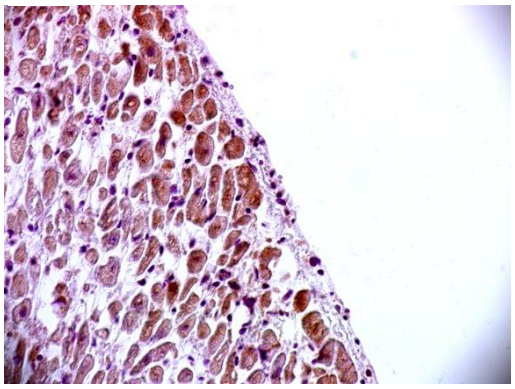

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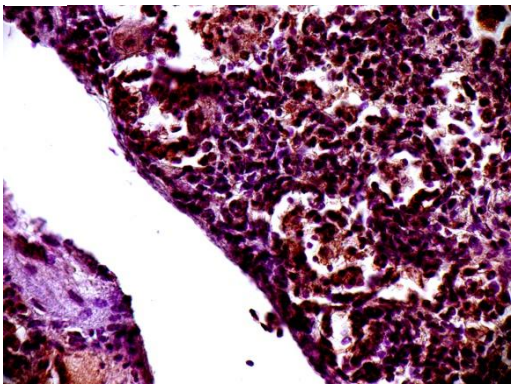

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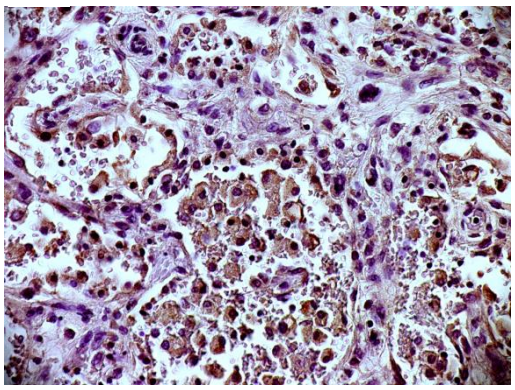

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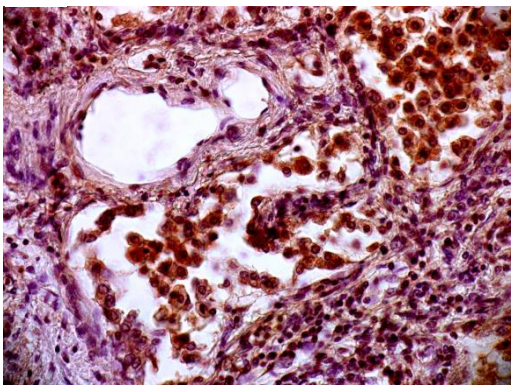

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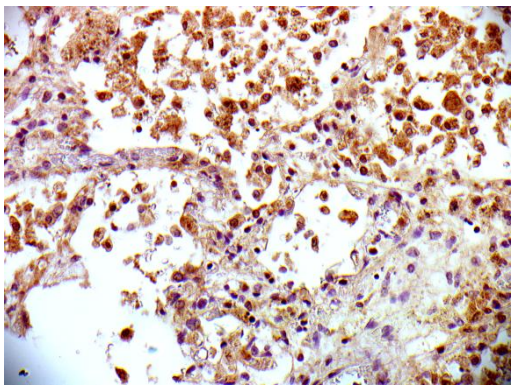
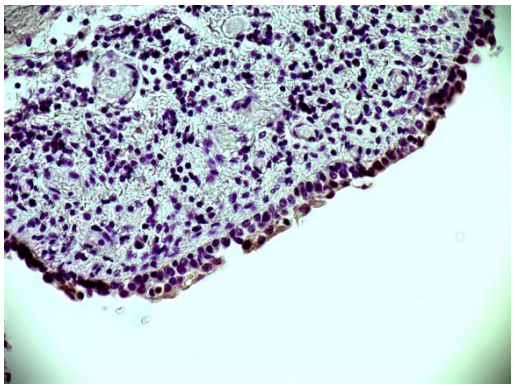


Figure 2-3. Immunohistochemical staining for IFN-γ of autopsy lung tissue from H1N1 infections.5μm sections of lung tissue where stained for IFN-γ using the chromogen diaminobenzedine (DAB) (brown) and counterstained with hematoxylin (blue).

A



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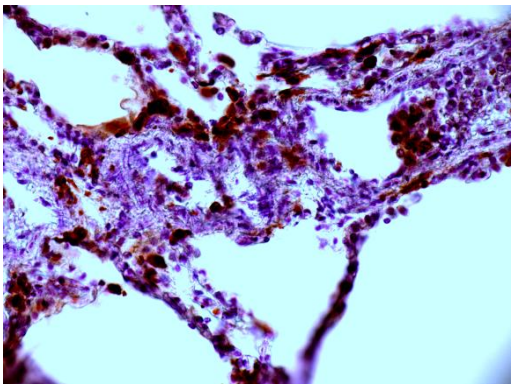

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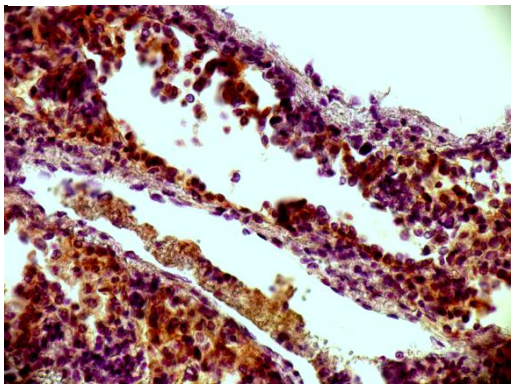

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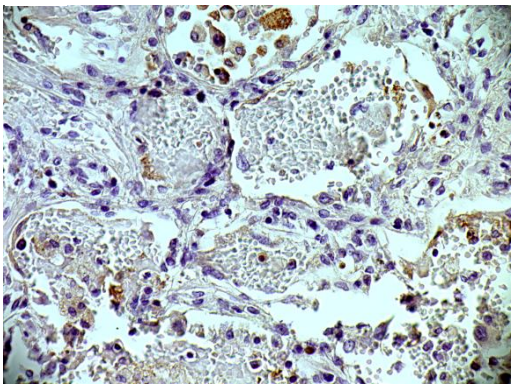

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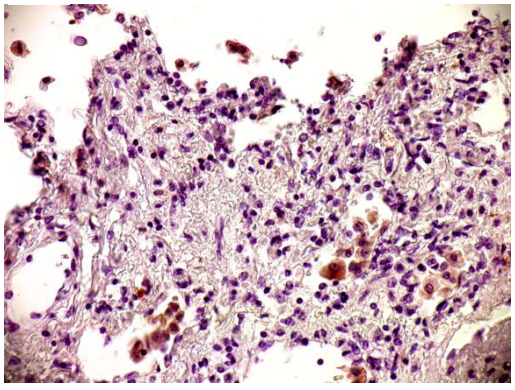

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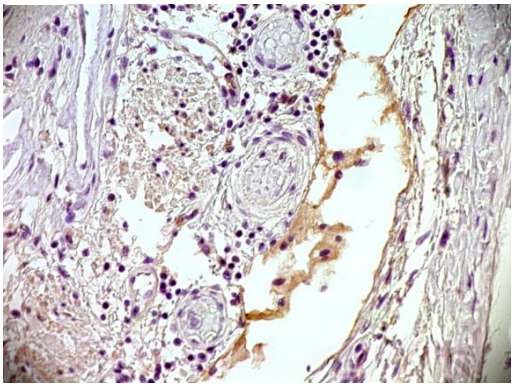

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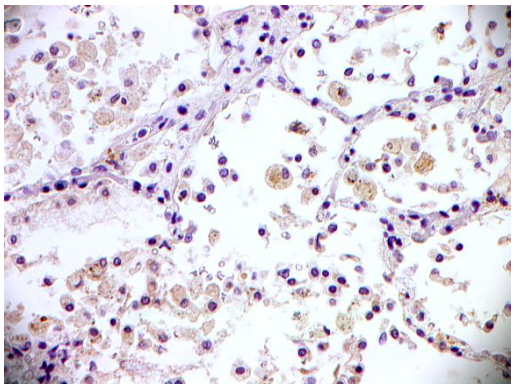
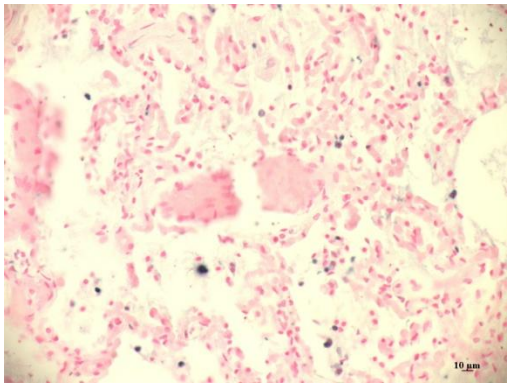


Figure 2-4. Immunohistochemical staining for IL-1β of autopsy lung tissue from H1N1 infections.5μm sections of lung tissue where stained for IL-1β using the chromogen diaminobenzedine (DAB) (brown) and counterstained with hematoxylin (blue).

TUNEL positive tissues demonstrated extensive cell death localized to the interstitium of consolidated areas of tissue. The vast majority of TUNEL positive cells were mononucleated leukocytes. Other cell types positive for TUNEL included fibroblasts and to lesser extent endothelial cells. The alveolar epithelial cells were primarily TUNEL negative in the vast majority of the sections (Figure 2-5)

A



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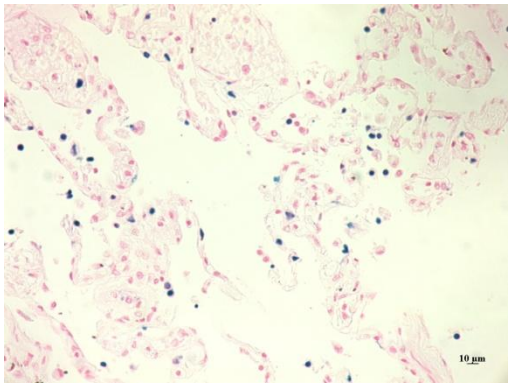

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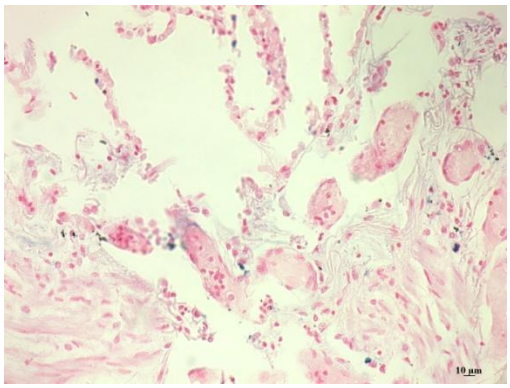

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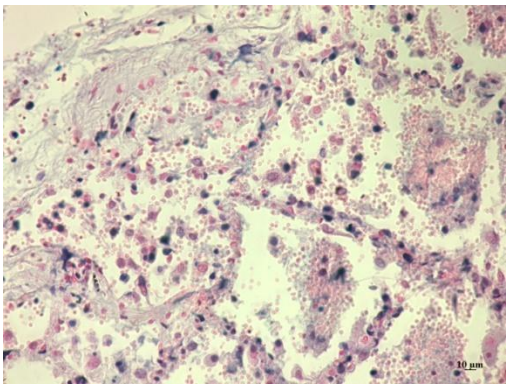

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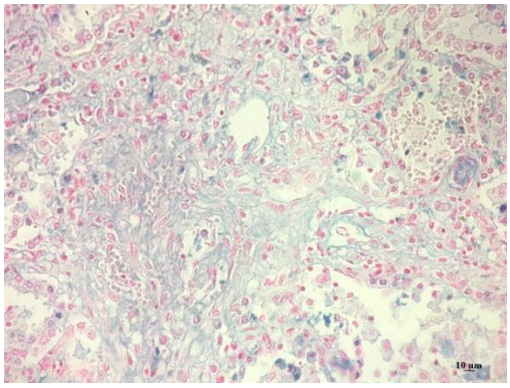

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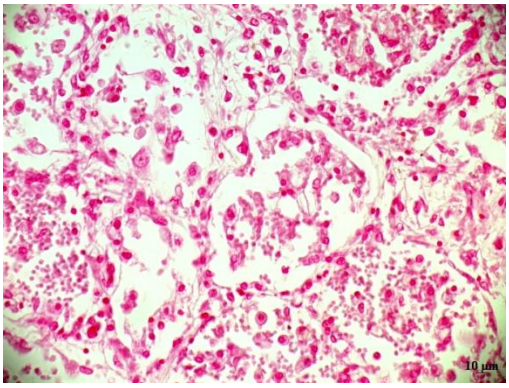

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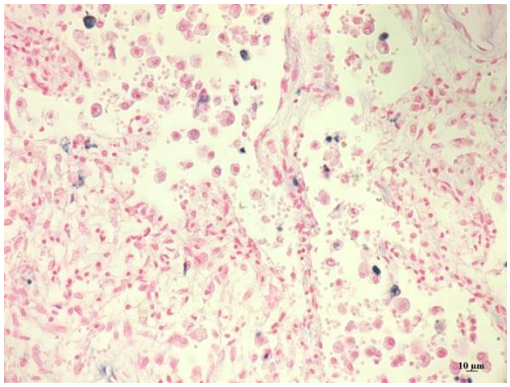


Figure 2-5. TUNEL assay on autopsy lung tissue from H1N1 infections**.** TUNEL assay was performed on 5µm sections of lung tissue from adults who succumb to pandemic H1N1 2009 infections. A-G corresponds to cases 1-7. Blue nuclei indicate TUNEL positive cells. Cells were counterstained with Nuclear Fast Red.

No histological evidence of secondary bacterial infection was seen in any of the cases, and no pathogenic bacteria were cultured from the autopsy tissue. Viral cultures performed on lung tissue obtained at the time of autopsy were positive in case 1, and negative in all other cases. There was no evidence of pulmonary embolism in any of the cases.

2.4 Discussion

This autopsy study reflects fatalities due to pandemic influenza A H1N1 (2009) in Manitoba, Canada during the spring and fall of 2009. Several studies have already noted remarkable aspects of this epidemic in Canada [273]. The age of those who become severely ill and those who die is significantly younger than seen in fatalities due to seasonal influenza. There is preponderance of women (6/7 deaths) and an association with the presence of underlying disease (4/5 non pregnant cases), and pregnancy (2/7, both in 3rd trimester), factors previously noted in an analysis of ICU-requiring pH1N1 cases in Canada [273]. The presence of two women in the 3rd trimester of pregnancy is concordant with data suggesting that pregnant women may be at increased risk for severe complications H1N1 influenza [273,275]. That 5 of 7 deaths occurred among individuals who were of Native Canadian ethnicity, appears to reflect the disproportionate fraction of patients with that background in the Manitoba cohort of severely ill individuals rather than a specific vulnerability of that population to poor outcomes. However, further research will clearly be required to determine whether aboriginals may have an increased predisposition to either acquiring infection or developing severe manifestations of infection.

Cases 1 and 2 are of particular interest, since they represent fulminant disease which was rapidly fatal within one to several days of onset, and provide an insight into the earliest pathology in the lung. Data on such early deaths is scant. The main finding was marked capillary vascular congestion and alveolar hemorrhage. Frothy bloody fluid was noted clinically antemortem and by clinicians during initial intubation in case 2, and was evident within the trachea and bronchi at the time of autopsy in both cases. Clinical descriptions of severe influenza pneumonia from the current epidemic as well fatal cases from the 1918 pandemic also described frothy pink or

bloody sputum [276,277]. The presence of vascular congestion and perivascular infiltrates suggests that a perturbation in the pulmonary vasculature at the level of the capillary may play a significant role early in disease.

The pathology of the lung (with the exception of cases 1 and 2) reflects a non-specific reaction pattern of alveolar injury ("acute lung injury") and repair ("organization"). While these finding are consistent with primary viral pneumonia, there are no specific pathological markers for viral etiology (such as viral inclusions) based upon tissue examination. The diagnosis requires correlation with clinical case definition and confirmatory laboratory studies such as viral culture or identification of viral antigens/nucleic acids. The spectrum of findings in the lungs in these cases is qualitatively similar to the pathology described in the H1N1 influenza pandemic of 1918, the smaller pandemics in 1957 and 1968 and in seasonal influenza [276,278-281].

The data also suggest an evolution of the dominant pulmonary pathology with increasing durations since illness onset. The cases where deaths occurred early demonstrated marked capillary and vascular congestion with alveolar hemorrhage. Tissues from deaths occurring in the 2nd week tended to demonstrate increasing frequency of hyaline membranes and diffuse alveolar damage with pneumocyte hyperplasia. Late deaths demonstrated evidence of organizing injury. In the one patient who died after 53 days, extensive airspace fibroplasia was seen. In short, specific findings will depend upon how quickly death has supervened, along the spectrum of initial infection and tissue damage. With the availability of intensive care and longer survival of severe lung injury, various degrees of tissue repair reaction are observed.

Bacterial pneumonia is well recognized to supervene in many cases of viral pneumonitis and to be associated with rapid deterioration and death. Many, if not most of the deaths, during the

1918 pandemic were ascribed to secondary bacterial infection and similar evidence of pulmonary superinfection has been described in more recent pandemics including the current one [281-285]. Interestingly, none of the cases examined had any evidence of secondary bacterial pneumonia.

Classic studies of severe influenza viral pneumonia have demonstrated consistent findings of early injury including interstitial edema with inflammatory infiltrates, capillary and small vessel thrombosis, hyaline membrane formation, variable acute intra-alveolar edema/hemorrhage, fibrin deposition, diffuse alveolar damage and frequently necrotizing tracheitis/bronchitis and bronchiolitis [276,286]. Later stages typically have shown organization of alveolar injury, with fibrosis, epithelial regeneration and squamous metaplasia. Findings described in previous autopsy studies include tracheitis and necrotizing bronchiolitis, which were not identified in the current cases. Bronchiolitis was not a striking feature in any of the cases, although mild peribronchial and peribronchiolar infiltrates were identified. Thrombosis is mentioned in previous autopsy studies [276,286]. Focal thrombosis was identified in a few cases, however since in-situ thrombi within pulmonary parenchymal arterioles are an expected finding in acute lung injury of any cause, this cannot be assumed to be a specific finding in influenza pneumonia. One report of severe pH1N1 pneumonia suggested a high frequency of pulmonary emboli [287]. No pulmonary emboli were seen in this autopsy series.

In all of the cases studied, some detachment of bronchial epithelium was seen. In the opinion of the authors this is most likely an artefact of autopsy material. Since surgical biopsy is generally not indicated in clinical cases of influenza, descriptions of findings from specimens other than autopsy tissue are very limited. Yelandi and Colby [288] described six open lung biopsies in patients subsequently diagnosed with influenza pneumonia. The one case was ultimately fatal demonstrated necrotizing bronchiolitis and diffuse alveolar damage with hyaline membranes.

Of the remaining patients (four who recovered and one lost to follow up), three demonstrated milder organizing acute lung injury; and two had a bronchiolitis-obliterans organizing pneumonia (BOOP)/cryptogenic organizing pneumonia (COP). Desquamation of bronchial mucosa was not noted.

TUNEL assay demonstrated interstitial leukocyte cell death. Monocytes and macrophages have been shown to induce apoptosis in influenza infected lymphocytes in a Fas dependent manner [288]. In addition influenza infected neutrophils also undergo apoptosis in a Fas dependent manner [289]. The expression of 3 pro-inflammatory cytokines was identified in the lung tissue from H1N1 infected adults. These cytokines play important roles in antiviral response and inflammation. TNF-α has been shown to be an important cytokine induced during influenza A infections [290] possessing antiviral activities. In addition this cytokine has been shown to prime lung epithelial cells to increase the production of chemokines necessary for the recruitment of leukocytes to the lung [291]. TNF-α also upregulates antiviral components of the TLR and RIG-I pathways both of which play a major role in the antiviral response to influenza A infections [292]. TNF-α can be membrane bound or cleaved to form a soluble form. The soluble form of TNF-α has been demonstrated to be necessary for coordinating an effective immune response against influenza A and lack of this form of TNFα resulted in a dysregulated immune response leading to worsening lung injury [293].

In conclusion, the lung morphology in seven fatal cases of H1N1 pneumonia has been described. The pathological findings are non-specific but consistent with influenza reflecting acute lung injury and repair and are qualitatively similar to previous descriptions of autopsy tissues during previous pandemics and with seasonal influenza. A progressive sequence of histopathological features has been classically observed and is reproduced in this series. That sequence includes

early vascular congestion and alveolar hemorrhage followed by the formation of hyaline membrane and diffuse alveolar damage with pneumocyte hyperplasia. In later stages, evidence of organization of injury and fibrosis is seen in fatal cases.

Chapter 3: H1N1 Septic Sera Characterization and Cellular Signalling in Fibroblasts

3.1 Introduction

Viral pneumonia induced by pandemic H1N1 infection is a major complication from this infection. It has the potential to lead to the more serious ARDS and death. Autopsy studies of pandemic H1N1 infected adults shows significant damage to the lungs. This damage is believed to result from both the replicating virus and the host immune response to the infection. Massive infiltrating leukocytes, dysfunction of the blood air barrier from increased permeability of the endothelium and loss of alveolar epithelial cells implicate cell death as a major contributor to lung dysfunction induced by pandemic H1N1 pneumonia. The host immune system utilizes apoptosis to clear infected cells and proteins from influenza A have been shown to modulate apoptosis as well. The downstream transcription factor targets from the various induces of apoptosis is an important point of cell signalling. STATs have been shown to play an important role not only in antiviral defense but also in broadly regulating apoptosis.

The STAT family is comprised of seven members: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. STAT family members are characterized by a highly conserved STAT homology (SH2) domain that facilitates dimerization via interactions between phosphotyrosine residues. Differences in the SH2 domain determine which of the STAT proteins will be recruited to a particular receptor. Each protein also contains a highly conserved tyrosine residue in the C-terminal region that is phosphorylated upon activation. The DNA binding domain is located

centrally and there is typically an oligomerization domain in the C-terminal region. STATs reside in the cytoplasm in a hypophosphorylated (inactive) form until phosphorylated by a Janus Activation Kinase (JAK). JAKs are receptor associated tyrosine kinases constitutively bound to cytokine and hematopoietin receptors. There are four members of the JAK family: JAK1, JAK2, JAK3 and TYK2. JAK1, JAK2 and TYK2 are ubiquitiously expressed but JAK3 has limited distribution.

The JAK/STAT pathway is essential in the biological response to cytokines. Binding of a cytokine ligand to its cognate receptor triggers dimerization bringing two JAK proteins into immediate proximity. Trans-auto-phosphorylation of the JAK proteins leads to activation and phosphorylation of the associated receptors. This induces a conformational change in the receptors that create a high-affinity docking site for STAT proteins. STAT proteins are recruited and phosphorylated by the JAKs after which they have a higher affinity for each other causing dimerization and dissociation from the receptor complex. No further modification is necessary before the STAT dimer is able to translocate to the nucleus and mediate transcription.

Mutant cell lines were created from HT1080 cells that lack specific Jak and STAT family members. 2fTGH cells showed no defect in IFN signalling, U1A cells lack Tyk2, U2A cells lack p48, U3A cells lack STAT1, U4A cells lack Jak1, U5A cells lack IFN-α receptor, U6A cells lack STAT2 and γ2A cells lack Jak2. We have performed H1N1 septic sera experiments in HT1080, 2fTGH, U3A and U3AR cells. These experiments showed that STAT1 is required to mediate H1N1 septic sera induced apoptosis.

3.2 Methods

3.2.1 Human Sera: Serum was derived from 8 patients who were in sepsis as defined by modified ACCP/SCCM Consensus Conference criteria induced by H1N1 influenza A infection. All pandemic H1N1 infections were PCR confirmed. Patients were required to have all (rather than a minimum of two) of the following four criteria for systemic inflammatory response syndrome 1) a body temperature greater than 38^{o}C or less than 36^{o}C; 2) a heart rate greater than 90 beats per minute; 3) tachypnea, manifested by a respiratory rate greater than 20 breaths per minute or hyperventilation, as indicated by a $PaCO_2$ of less than 32 mm Hg; 4) an alteration in the white blood cell count (i.e. WBC greater than 12,000/ mm^3, less than 4,000/mm^3, or the presence of more than 10% immature neutrophils). Serum samples were obtained within 48 hours of admission. Human septic serum was obtained after informed consent under an approved Institutional Review Board (University of Manitoba) protocol. Subjects contributing HSS for this study were not known to have pre-existing structural heart disease. 10 cc of blood was drawn from the patient and centrifuged for 10 min at 1700xg. The supernatant representing the serum was aliquoted and stored at -70^{o}C. Normal human serum was obtained from commercial sources (Sigma Laboratories).

3.2.2 ELISA: Human septic and normal serum concentrations of TNF-α, IL-1β and IFN-γ were determined by ELISA. The assay is based on an ELISA with Streptavidin conjugated to HRP and TMB was the substrate used. For the detailed protocol, refer to the DuoSet ELISA Development System on the website www.RnDSystems.com or in the following product manuals: Human TNF-α/TNFSF1A, Catalog Number: DY210; Human IL-1β/IL-1F2, Catalog Number: DY201; and Human IFN-γ, Catalog Number: DY285. The following modifications

were made to the ELISA protocol. Four washes were performed instead of three in order to reduce the background. Furthermore, the reagent diluents were optimized for the quantification of each cytokine. The diluents consisted of 10% FCS with PBS, 2% FCS with PBS and 0.5% FCS with PBS for TNF-α, IL-1β and IFN-γ, respectively.

3.2.4 Trypan Blue Exclusion Assay**:** HT1080, 2fTGH, U3A, U3A R cells (5 x 10^4 cells/96 well plate) were treated with 5% uninfected sera, 5% H1N1 infected sera in DMEM as indicated. After 12 h, the cells were trypsinized and viable cells counted using trypan blue exclusion assay. The caspase 3, -6, -7, -8, -10 inhibitor Ac-DEVD-CHO; (Calbiochem; caspase inhibitors were prepared in DMSO at a stock concentration of 10 mM and used at a concentration of 10 μM in tissue culture) was prepared in ethanol at a stock concentration of 50 mg/mL and used at a concentration of 100μM in tissue culture were added individually at the same time as the 5% normal or 5% H1N1 infected serum.

3.2.5 TUNEL Assay**:** TUNEL assay was performed on HT1080, 2fTGH, U3A and U3AR cells using the R&D systems/Trevigen HT TiterTACS (Trevingen Apoptotic Cell System) colourimetric assay. Refer to catalogue number 4822-96-K. Cells seeded at 5X10^4 cells/well were treated with either 5% uninfected serum or 5% H1N1 infected serum for 12 hours. Cells were fixed in plate with 3.7% buffered formaldehyde (in PBS) for 10 minutes, washed in PBS fixed with methanol for 20 minutes washed in PBS and then labelled according to manufacturer's protocol. The reaction was read on a Fluostar Optima plate reader (BMG Labtech).

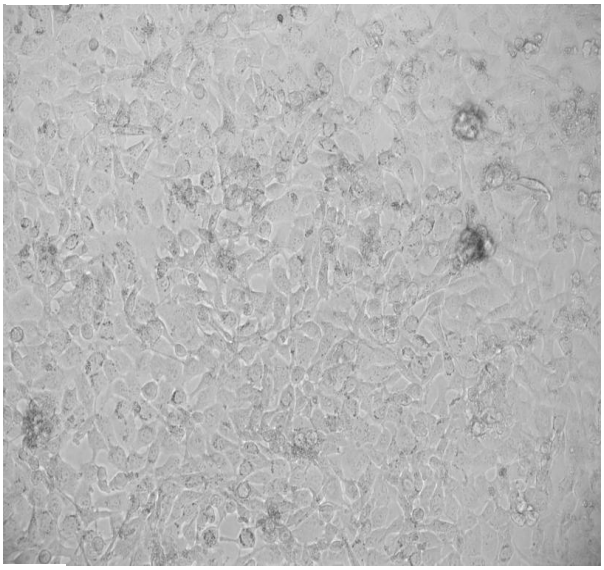
3.2.6 Caspase 3 Assay**:** Caspase 3 assay was performed on HT1080, 2fTGH, U3A and U3AR cells using the Cell Signalling Caspase 3 Activity Assay Kit (#5723). Cells were seeded in a 96

well plate at $5X10^4$ cells/well. The cells were treated with either 5% uninfected serum or 5% H1N1 infected serum for 12 hours. Next the cells were lysed on plate using supplied lysis buffer and assayed according to manufacturer's protocol. Fluorescence was read on a Fluostar Optima plate reader (BMG Labtech).

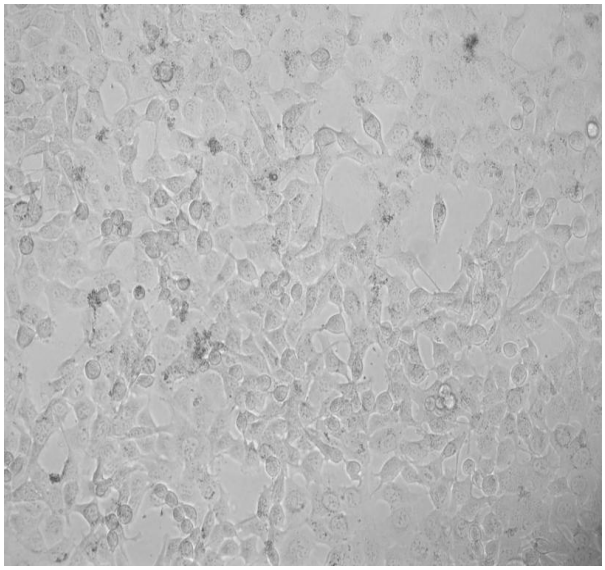
3.3 Results

Sera from H1N1 infected adults has the ability to induce cell death in fibroblast cells HT1080, 2fTGH and U3AR1 cells compared to sera from uninfected adults (Figure 3-1 A,B,D,E,F,H). U3A cells show resistance to H1N1 infected sera induced cell death (Figure 3-1G).

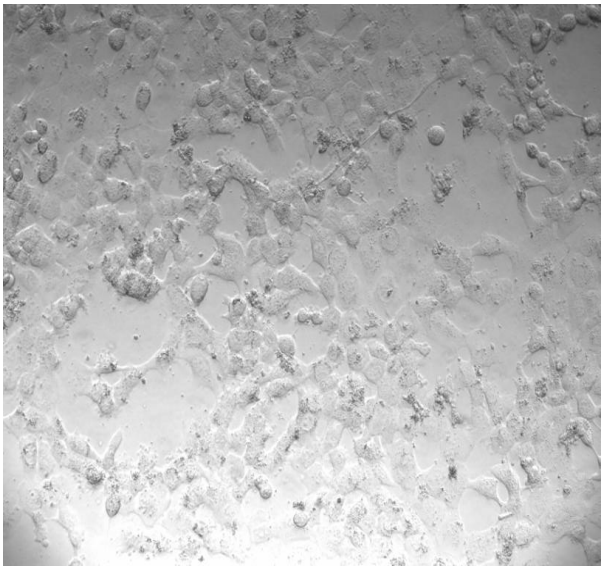
A HT1080



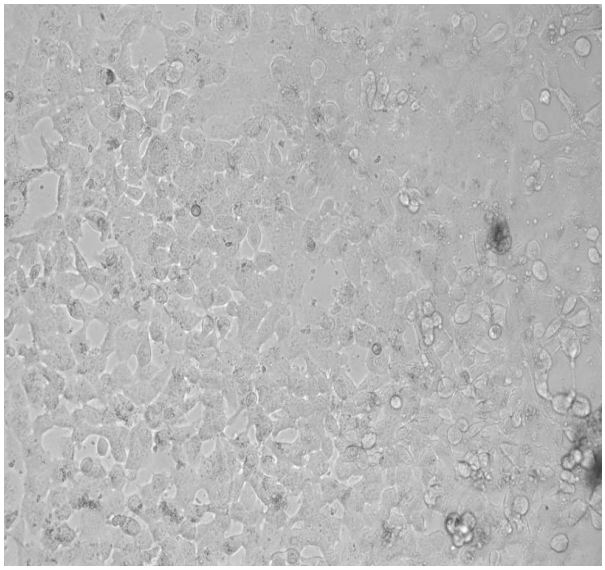
B 2fTGH



C U3A



D U3A R1



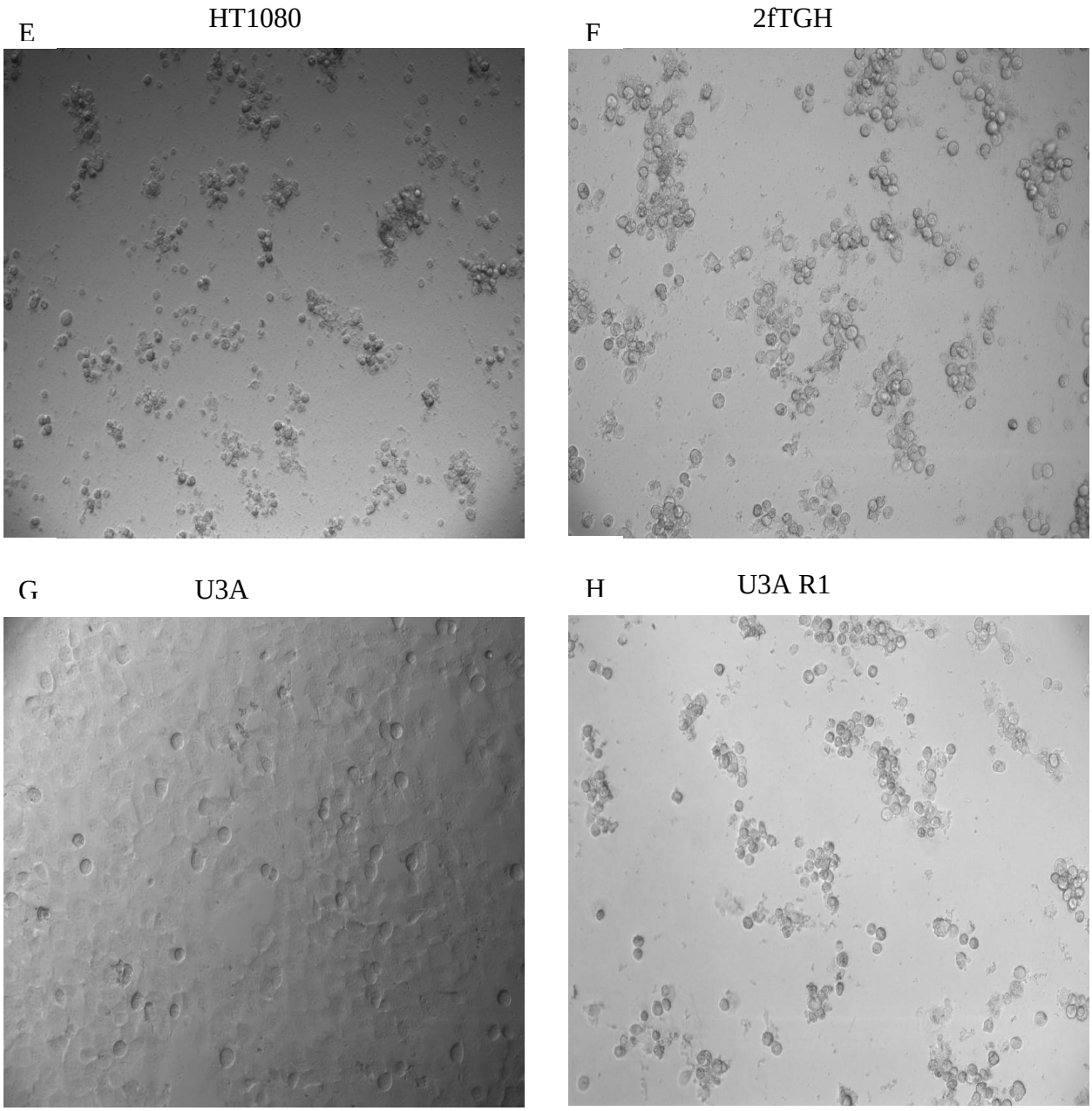


Figure 3-1. Light micrographs of fibroblasts treated with uninfected and H1N1 infected sera.
Fibroblast cells were seeded in a 96 well plate at a density of $5X10^4$ cells per well. Cells were treated with either 5% uninfected sera or 5% H1N1 infected sera for 18 hours. A-D fibroblasts treated with uninfected sera, E-H cells treated with H1N1 sera.

H1N1 sera had elevated levels of the cytokines TNF-α, IFN-γ and IL-1β (Table 3-1). The H1N1 sera had an average of 113 pg/mL, 49 pg/mL and 26 pg/mL for TNF-α, IFN-γ and IL-1β respectively while the sera from uninfected adults had undetectable levels of these cytokines.

Table 3-1. Serum concentration of cytokines from fatal cases of pandemic H1N1 infections.

Sample ID	TNF-α (pg/mL)	IFN-γ (pg/mL)	IL-1β (pg/mL)
H1	21	13	15
H2	18	26	16
H3	65	33	48
H4	72	17	34
H5	58	22	71
H6	229	47	63
H7	87	14	54
H8	351	39	92
N1	0	0	0
N2	0	0	0
N3	0	0	0
N4	0	0	0
N5	0	0	0
N6	0	0	0
N7	0	0	0
N8	0	0	0

Serum concentrations for TNF-α, IFN-γ and IL-1β were determined for 8 adults infected with pandemic H1N1 influenza A (H), and 8 uninfected adults (N) using sandwich ELISA. Values are averages from duplicate readings. 0 indicates below detection limit.

H1N1 sera induced apoptosis in HT1080 and 2fTGH cells as determined by trypan blue 23% for both cell lines (Figure 3-2). TUNEL assays indicates H1N1 infected sera induced DNA breaks in HT1080 and 2fTGH cells to a greater degree then uninfected sera (Figure 3-3). U3A cells were resistant to H1N1 induced apoptosis (Figures 3-2, 3-3) decreasing the levels of dead cells to levels slightly greater then the uninfected sera (see appendix for individual sera results). H1N1

sera induced apoptosis in U3A rescues cells (U3AR) to levels of the parental lines HT1080 and 2fTGH cell lines which implicates transcription factor STAT1 in mediating H1N1 sera induced apoptosis.

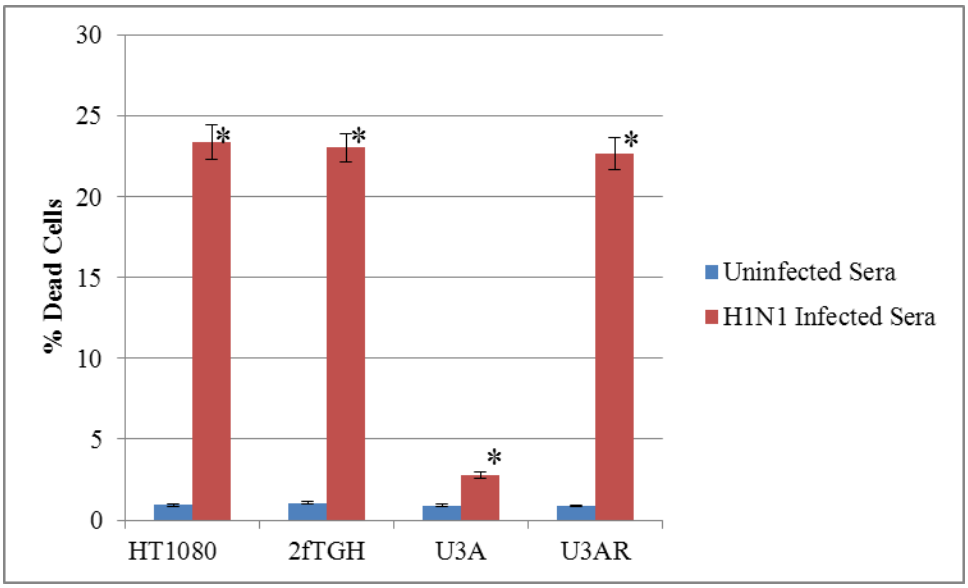


Figure 3-2. Trypan blue staining of fibroblast cell lines treated with sera from H1N1 infected and uninfected sera. Fibroblast cell lines HT1080, 2fTGH, U3A and U3AR were seeded in 96 well plates at a density of $5X10^4$ per well and treated with either 5% H1N1 infected sera or 5% uninfected sera for 12 hours before staining. Values plotted are avereages of duplicate counts expressed as percent dead cells. Error bars represent standard error of the mean. * $p<0.05$ t-test for uninfected vs H1N1 infected sera by cell type

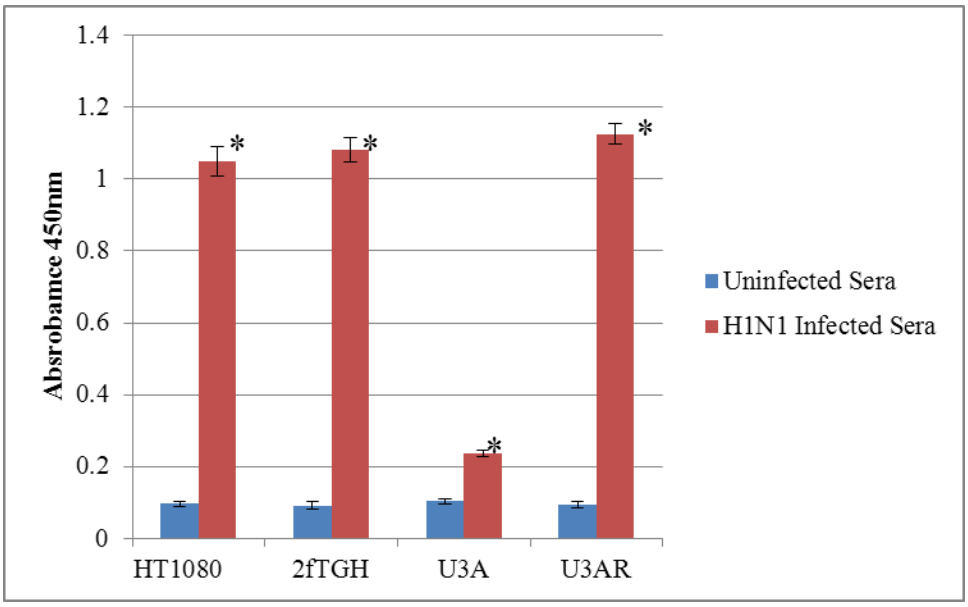


Figure 3-3. TUNEL assay of fibroblast cell lines treated with sera from H1N1 infected and uninfected sera. Fibroblast cell lines HT1080, 2fTGH, U3A and U3AR were seeded in 96 well plates at a density of $5X10^4$ per well and treated with either 5% H1N1 infected sera or 5% uninfected sera for 12 hours before staining. Values plotted are avereages of duplicate counts expressed as percent dead cells. Error bars represent standard error of the mean. * $p<0.05$ t-test for uninfected vs H1N1 infected sera by cell type

H1N1 sera treatment of HT1080 and 2fTGH cells led to the activation of caspase 3 as determined by caspase 3 colourimetric assay (Figure.3-3) compared to uninfected sera. Caspase 3 activation was greatly reduced to levels slightly greater then uninfected sera in U3A cells treated with H1N1 sera as compared to parental lines HT1080 and 2fTGH (see appendix for individual serum results). Caspase 3 activation was restored in U3AR cells to levels comparable to the parental cell lines implicating STAT1 in mediating caspase 3 activation.

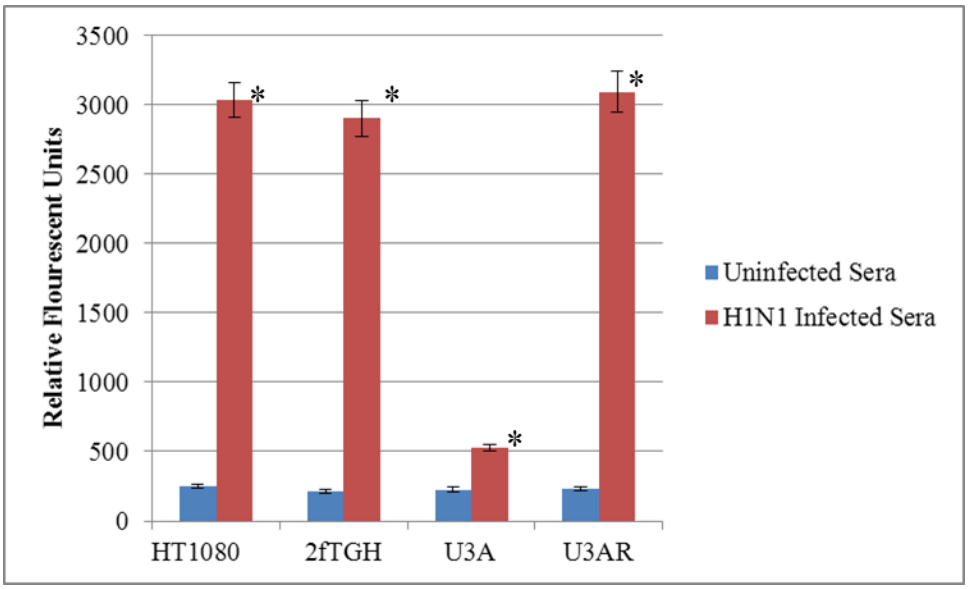


Figure 3-4. Caspase 3 activity assay of fibroblast cell lines treated with sera from H1N1 infected and uninfected sera. Fibroblast cell lines HT1080, 2fTGH, U3A and U3AR were seeded in 96 well plates at a density of $5X10^4$ per well and treated with either 5% H1N1 infected sera or 5% uninfected sera for 12 hours before assaying for caspase 3 activity. Values plotted are avereages of duplicate counts expressed as relative fluorescent units. Error bars represent standard error of the mean. * $p<0.05$ t-test for uninfected vs H1N1 infected sera by cell type

Treatment of HT1080, 2fTGH and U3AR cells with the caspase 3 inhibitor Ac-DEVD-CHO reduced H1N1 sera induced apoptosis in these cell lines (Figure 3-5) (see appendix for individual sera results).

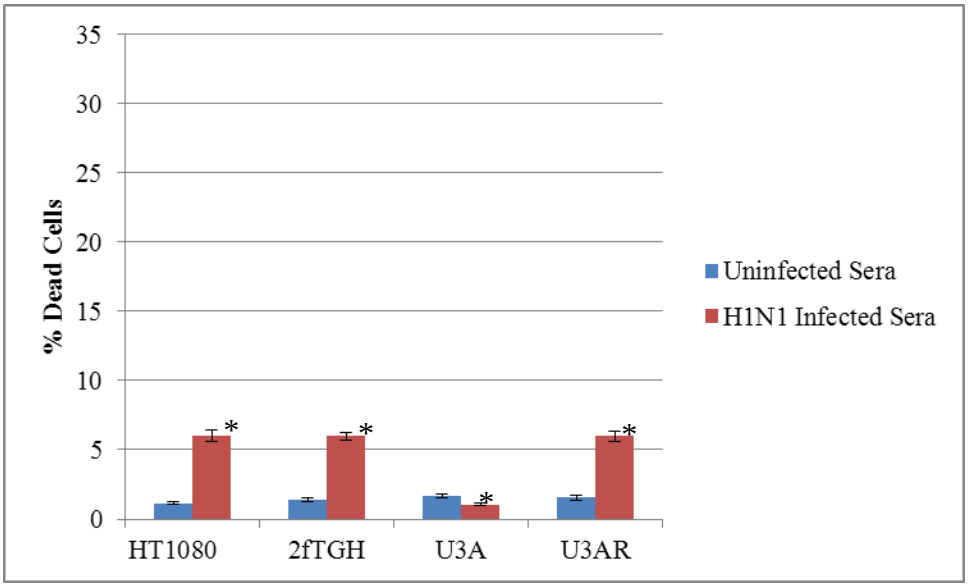


Figure 3-5. Trypan blue staining of fibroblast cell lines treated with caspase inhibitor and sera from H1N1 infected and uninfected sera. Fibroblast cell lines HT1080, 2fTGH, U3A and U3AR were seeded in 96 well plates at a density of $5X10^4$ per well and treated with caspase 3 inhibitor Ac-DEVD-CHO and either 5% H1N1 infected sera or 5% uninfected sera for 12 hours before staining. Values plotted are avereages of duplicate counts expressed as percent dead cells. Error bars represent standard error of the mean. * $p<0.05$ t-test for uninfected vs H1N1 infected sera by cell type

3.4 Discussion

In this study it has been shown that the sera from pandemic H1N1 infected adults contain high levels of the cytokines TNF-α, IFN-γ and IL-1β (Table 3-1). These levels are higher than those found in sera of adults with bacterial induced sepsis [294]. Pandemic strains of influenza A have the potential to illicit stronger inflammation and cytokine production since the population generally has little or no immune exposure to those strains. This potentiates a higher probability of inducing a hyper inflammatory state in those infected. In addition the virulence of the strain of influenza A and the viral load induces a stronger inflammatory response. Influenza A has evolved mechanism to antagonize the antiviral response yet allows for the continued production of inflammatory cytokines. This combination potentiates the developed of influenza A induced sepsis.

The sera from H1N1 infected individuals demonstrated the ability to induce apoptosis in treated fibroblast cells. A recent study in mice demonstrated that injury to the cornea from lasers induced the expression of TNF-α, IL-1β and IFN-γ. They attributed these cytokines to the induction of apoptosis of the endothelium of the cornea demonstrating the apoptotic potential of these inflammatory cytokines [295]. In addition to the apoptotic inducing signalling by TNF-α other members of the TNF family have been shown to be important mediators of viral clearance. Fas ligand and TRAIL are both mediators of apoptosis utilized by the immune system to kill influenza A infected cells. The presence of other inflammatory cytokines would also have a contribution to the apoptosis inducing ability of the H1N1 infected sera as it has been shown that cytokines can work synergistically and induce the activation of similar transcription factors which regulate the inflammatory and apoptotic processes.

The observation that U3A cells are generally resistant to the apoptotic effects of H1N1 infected sera implies a role for STAT1 in mediating the apoptotic signalling. It has been shown that IFN-γ induced activation of STAT1 mediates apoptosis in A431 cells [296]. They also demonstrated that this induction of apoptosis requires STAT1 induced expression of caspase1. The requirement for STAT1 in mediating TNF-α induced apoptosis has been demonstrated [297]. The inflammatory effects of IFN-γ can be amplified by the presence of other mediators in the sera. Gao et al demonstrated the synergistic effect of IL-17 on IFN-γ induced production of iNOS in macrophages [298]. This effect was mediated by STAT1 further implicating the importance of STAT1 not only in mediating apoptosis but in mediating inflammatory processes.

3.5 Conclusion

Sera from pandemic H1N1 infected adults contain inflammatory mediators. In addition the sera possess the ability to induce apoptosis in fibroblast cells. This process is mediated in part by STAT1 making this a potential target in understanding the cellular injury that occurs during influenza infections.

Chapter 4: Characterization of Factors in Human Septic Sera that Mediate Cellular Dysfunction

4.1 Introduction

Sepsis and septic shock are the leading cause of morbidity and mortality in noncoronary intensive care units worldwide [299-301]. Annually, sepsis develops in approximately 800,000 people with a mortality rate that approaches 50% [299-301] and the yearly frequency of sepsis incidence has been increasing by 9% per year [1,301]. During bacterial sepsis the release of bacterial endotoxins and exotoxins induce the cellular production and release of pro-inflammatory cytokines which include tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β and interferon (IFN)-γ. This is followed by production of chemokines, which are small chemotactic cytokines, such as growth-related oncogene (GRO), monocyte chemo-attractant protein (MCP)-1, and IL-8 [302,303]. At a later stage, anti-inflammatory cytokines such as IL-10 and IL-5 are produced. The complex expression of cytokines and chemokines mediates the process of inflammation [303]. The deregulation of the production of pro-inflammatory, anti-inflammatory or chemokines mediators can lead to damage to the host.

Previous studies, which have targeted individual cytokines and chemokines during sepsis, have not been successful to date [304,305]. The use of activated protein C (APC) is the only approved sepsis therapy which modulates the host cytokine responses. Using antibody blot array technologies we can specifically identify sera factor profile during sepsis and septic shock. In addition, by assessing the key cytokine, chemokine and growth factor expression levels, it will

be possible to initiate therapies that modulate the host response at the most advantageous time during the course of the disease.

Using antibody blot arrays, we have determined the differential cytokine, chemokine and growth factor expression profile comparing four normal sera to four septic sera. We observed that the levels of key cytokines, chemokines and growth factors were differentially expressed in the sera of septic patients as compared to the sera of healthy individuals. The pro-inflammatory cytokines including, TNF-α, IL-1β, IFN-γ, IL-7 and IL-15, were increased in the sera of septic patients when compared to the sera from healthy volunteers. In addition the anti-inflammatory cytokines IL-5, IL-10, and IL-13; the chemokines MCP-1, MCP-2, IL-8, GRO, GRO-α, TARC, SDF-1, MIG and MIP-1δ; and other factors such as leptin, angiogenin, EGF and PDGF-BB showed differential expression levels in the sera of septic patients when compared to the sera of healthy individuals. These factors were also differentially regulated in the four individual septic sera. TNF-α was the only factor that showed elevated levels in all septic sera. Using antibody blot array technologies, key factors in septic sera will be identified and targeted for therapeutic interventions.

4.2 Methods

4.2.1 Study Patients from whom HSS was obtained**.** Serum was derived from 4 patients who were in acute phase of septic shock as defined by modified ACCP/SCCM Consensus Conference criteria. Patients were required to have all (rather than a minimum of two) of the following four criteria for systemic inflammatory response syndrome 1) a body temperature greater than 38^{o}C or less than 36^{o}C; 2) a heart rate greater than 90 beats per minute; 3) tachypnea, manifested by a respiratory rate greater than 20 breaths per minute or hyperventilation, as indicated by a $PaCO_2$

of less than 32 mm Hg; 4) an alteration in the white blood cell count (i.e. WBC greater than 12,000/ mm^3, less than 4,000/mm^3, or the presence of more than 10% immature neutrophils). In addition, all patients whose serum samples were utilized exhibited positive blood cultures with a defined focus of infection (e.g. peritonitis or pneumonia) and required substantial pressor therapy (>0.5 ug/kg/min norepinephrine) to maintain mean arterial pressure > 65 mm Hg. Serum samples were obtained within 24 hours of presentation with septic shock. Human septic serum was obtained after informed consent under an approved Institutional Review Board (Rush University)-approved protocol. Subjects contributing HSS for this study were not known to have pre-existing structural heart disease. 10 cc of blood was drawn from the patient and centrifuged for 10 min at 1700xg. The supernatant representing the serum was aliquoted and stored at -70^oC. Before use in experiments the serum was heat inactivated at 56^oC for 45 min. Cytokine profiles of serum donors with septic shock is shown in Table 4-1. Normal human serum was obtained from laboratory personnel and commercial sources (Sigma Laboratories).

4.2.2 Reagents. Bradford dye reagent was and Bovine serum albumin (BSA) standards where purchased from BioRad (Mississauga, Canada). TNF-α, IL-1β and IFN-γ were analyzed by commercial Enzyme-Linked Immunosorbent Assay (ELISA) kits were purchased from R&D Systems (Minniapolis, United States). Tetramethylbenzidine (TMB) was purchased from Pierce (Rockford, United States) and Streptavidin conjugated to horseradish peroxidase (HRP) from R&D Systems (Minniapolis, United States). Fetal calf serum (FCS) was purchased from Sigma (Oakville, Canada) and the phosphate buffer saline (PBS) from Invitrogen (Burlington, Canada). The Human cytokine antibody array III kit was purchased from RayBiotech, Inc (Norcross, United States).

4.2.3 Bradford assay. Human septic and normal sera total protein quantification was performed using the Bradford assay. A standard curve was established using BSA with a concentration range from 0.2 µg/ml to 1.4 µg/ml. Absorbance at a wavelength of 595 nm was read. The absorbance of the sera were plotted on a standard curve to obtain the total protein concentration for each sample.

4.2.4 Sandwich ELISA assays. Human septic and normal serum concentrations of TNF-α, IL-1β and IFN-γ were determined by ELISA. The assay is based on an ELISA with Streptavidin conjugated to HRP and TMB was the substrate used. For the detailed protocol, refer to the DuoSet ELISA Development System on the website www.RnDSystems.com or in the following product manuals: Human TNF-α/TNFSF1A, Catalog Number: DY210; Human IL-1β/IL-1F2, Catalog Number: DY201; and Human IFN-γ, Catalog Number: DY285. The following modifications were made to the ELISA protocol. Four washes were performed instead of three in order to reduce the background. Furthermore, the reagent diluents were optimized for the quantification of each cytokine. The diluents consisted of 10% FCS with PBS, 2% FCS with PBS and 0.5% FCS with PBS for TNF-α, IL-1β and IFN-γ, respectively.

4.2.5 Human cytokine antibody array. The human cytokine antibody array protocol was followed. The detailed protocol can be found in RayBio® Human Cytokine Antibody Array Protocol, © 2004 RayBiotech Inc. or at http://www.raybiotech.com/manual/human_manual.pdf. Human septic and normal serum with 10 mg total protein was used to hybridize each membrane. Detection of signals on the membranes was performed using chemiluminescence and captured using BioRad ChemiDoc XRS imaging system.

4.2.6 Statistics. For the blot arrays, the background, which was measured as the averaged negative controls, was subtracted from the intensity of every spot on the array. In addition, the intensities were normalized to the positive controls for every blot. A one-way analysis of variance (ANOVA) was performed for the statistical comparison, followed by Tukey's test when suitable. A level of $p < 0.05$ was considered to be significant.

4.3 Results

4.3.1 ELISA protein quantification. The levels of TNF-α, IL-1β and IFN-γ in the serum of the four septic patients and in the serum of healthy individuals were quantified using the Enzyme-Linked Immunosorbant Assay (ELISA) kit from R&D Systems. Patient SE was a female of 81 years old that had *Staphylococcus aureus* as the infecting organism during sepsis. Patient SE succumbed to the disease. The levels of TNF-α, IL-1β and IFN-γ for this patient are 43.9 pg/ml, 6.1 pg/ml and 22.8 pg/ml respectively (Table 4-1). Patient SF a male of 73 years, with the infecting organism *Haemophilus influenzae* survived from sepsis. This patient had levels of TNF-α, IL-1β and IFN-γ of 13.4 pg/ml, 3.6 pg/ml and 6.8 pg/ml respectively. Patient SG was infected with *Escherichia coli*. This patient, a male of 68 years old also survived sepsis and the levels of cytokines measured from the serum of this patient were 19.0 pg/ml, 7.7 pg/ml and 10.3 pg/ml for TNF-α, IL-1β and IFN-γ respectively. The fourth serum SH was from a male of 54 years of age with *Staphylococcus pneumonia* as the initial infecting organism for sepsis. This patient also survived sepsis and had levels of 39.0 pg/ml, 1.8 pg/ml and 6.0 pg/ml for TNF-α, IL-1β and IFN-γ in his serum. Normal sera from healthy lab volunteers did not have measurable levels of TNF-α, IL-1β and IFN-γ.

Table 4-1: Demongraphic and Enzyme-linked Immunosorbant Assay Data

Patient	Gender	Age	Infecting organism	Outcome	TNF-α (pg/mL)	IL-1β (pg/mL)	IFN-γ (pg/mL)
SE	Female	81	Staphylococcus aureus	Died	43.9	6.1	22.9
SF	Male	73	Heamophilus influenzae	Survived	13.4	3.6	6.8
SG	Male	68	Escherichia coli	Survived	19.0	7.7	10.3
SH	Male	54	Streptococcus pneumoniae	Survived	39.0	1.8	6.0
Normal	Healthy individual				---	---	---

Categorization of sepsis using physiological parameters of four septic patients (SE, SF, SG, and SH), and cytokine levels, measured with an Enzyme-Linked Immunosorbant Assay (ELISA) using the sera from the four septic patients. The cytokine levels in the sera of four healthy volunteers were also measured (all normal serums quantified had non-detectable levels of cytokines).

4.3.2 Cytokine, chemokine and growth factor blot array. The cytokine, chemokine and growth factor profile of the sera of septic patient SE, SF, SG and SH and the healthy individuals NA, NB, NC and ND was performed using RayBiotech cytokine blots (Figure 4-1A and B). We performed an internal comparison of the cytokines between each healthy individual. There was no statistical differences between the cytokine levels from one serum of healthy individuals to another, therefore the data was averaged and used to compare the relative cytokine level from the serum of the four septic patients. Significant differences in the levels of many cytokines were observed in the serum of septic patients. TNF-α showed the greatest relative increase to normal serums and in addition, was the only cytokine that was commonly up-regulated in all septic serums (Table 4-2).

Figure 4-1A Cytokine Blot Array Map

	a	b	c	d	e	f	g	h	i	j	k	l
1	Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO-α	l-309	IL-1α	IL-1β
2	Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO-α	l-309	IL-1α	IL-1β
3	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10	IL-12	IL-13	IL-15	IFN-γ
4	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10	IL-12	IL-13	IL-15	IFN-γ
5	MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1δ	RANTES	SCF	SDF-1	TARC	TGF-β1
6	MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1δ	RANTES	SCF	SDF-1	TARC	TGF-β1
7	TNF-α	TNF-β	EGF	IGF-1	Angiogenin	Oncostain M	Thrombopoietin	VEGF	PDGF BB	Leptin	Neg	Pos
8	TNF-α	TNF-β	EGF	IGF-1	Angiogenin	Oncostain M	Thrombopoietin	VEGF	PDGF BB	Leptin	Neg	Pos

Figure 4-1B Cytokine Blot Array

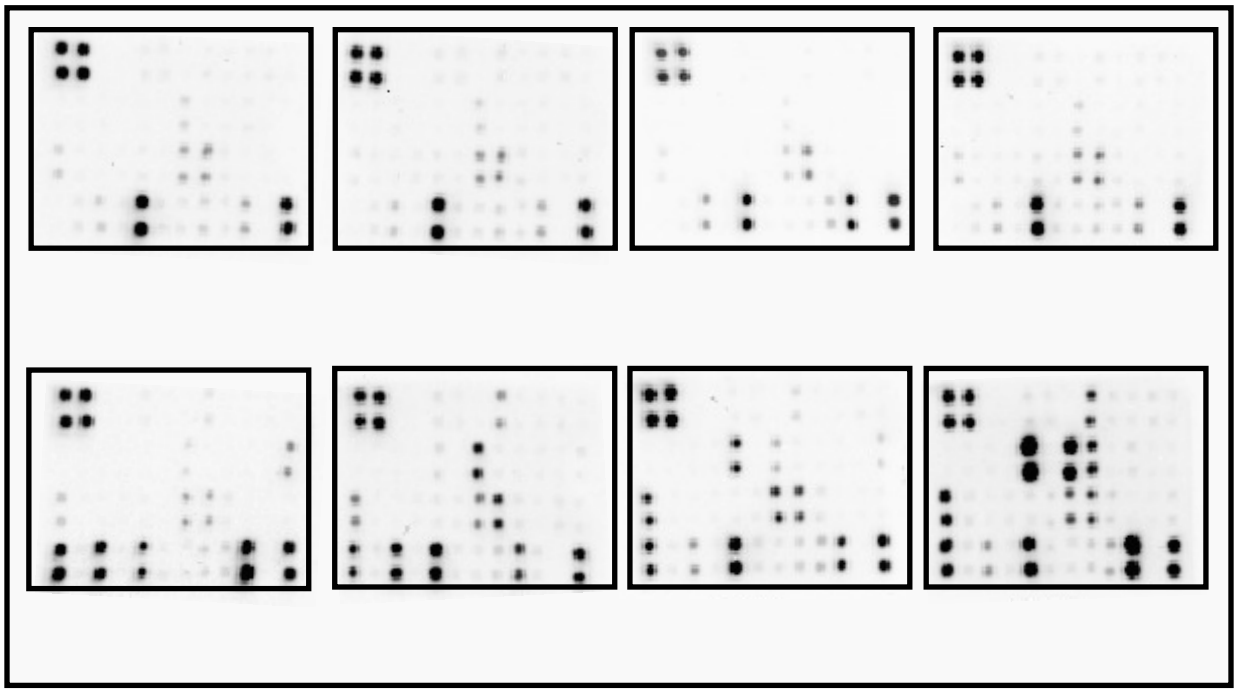


Figure 4-1A) Cytokine blot array grid, Pos; positive: Neg; negative: ENA-78; epithelial-derived neutrophil activating protein 78: GCSF; granulocyte colony stimulating factor: GM-CSF; granulocyte-macrophage colony stimulating factor: GRO; growth-related Oncogene: I-309; inflammatory cytokine I-309: IL; interleukin: IFN; interferon: MCP; monocyte chemo-attractant protein: MCSF; macrophage colony stimulating factor: MDC; macrophage-derived chemokine: MIG; monokine induced by gamma interferon: MIP; macrophage inflammatory protein: RANTES; regulated upon activation, normally T-expressed, and presumably secreted: SCF; stem cell factor precursor: SDF; stromal cell-derived factor 1: TARC; thymus and activation-regulated chemokine: TGF; transforming growth factor: TNF; tumor necrosis factor: EGF; epidermal growth factor: IGF-1; insulin-like growth factor 1: VEGF; vascular endothelial growth factor: PDGF BB; platelet-derived growth factor beta polypeptide BB. Figure4-1B) Cytokine blot array hybridized with serums from septic patients and healthy individuals.

Table 4-2: Cytokine Blot Array

Protein	Classification	Patient SE	Patient SF	Patient SG	Patient SH
Interferon γ	Pro-inflammatory	125.3	---	24.4	---
Interleukin 15	Pro- inflammatory	---	---	---	7.9
Interleukin 1 beta	Pro- inflammatory	---	---	736.7	---
Interleukin 7	Pro- inflammatory	---	---	---	149.6
Tumor necrosis factor α	Pro- inflammatory	1738.5	781.2	875.8	955.9
Interleukin 6	Pro- and anti- inflammatory	---	---	146.0	1188.2
Interleukin 10	Anti- inflammatory	---	---	---	126.1
Interleukin 13	Anti- inflammatory	---	---	---	4.8
Interleukin 5	Anti- inflammatory	---	---	27.9	19.2
Growth-related oncogene	Chemokine	---	10.8	2.9	22.0
Growth-related oncogene alpha	Chemokine	---	---	---	5.4
Interleukin 8	Chemokine	---	9.0	---	34.4
monocyte chemo-attractant protein 1	Chemokine	---	7.2	13.2	17.9
monocyte chemo-attractant protein-2	Chemokine	---	---	---	4.3
monokine induced by gamma interferon	Chemokine	---	---	---	3.8
macrophage inflammatory protein 5	Chemokine	---	---	2.4	---
stromal cell-derived factor 1	Chemokine	---	---	---	2.1
Thymus and activation-regulated chemokines	Chemokine	---	5.8	---	---
Angiogenin	Other cytokine	0.4		---	---
epidermal growth factor	Other cytokine	7.9	7.3	---	---
Leptin	Other cytokine	8.0	---	---	8.4
Platelet derived growth factor-BB	Other cytokine	---	7.1	---	---

Relative levels of protein expressed in the sera of septic patients compared to healthy individuals. --- indicates no difference between levels found in the septic sera when compared to the healthy sera.

4.4 Discussion

Cytokines, chemokines and growth factors play important roles at the molecular level during sepsis. They play a direct role, such as IL-1β which can induce apoptosis or an indirect role such as chemokines which attract leukocytes to the site of infections which in their turn release other mediators of inflammation to further activate the host response to inflammation. By determining the expression patterns and levels of key cytokines, chemokines and growth factors we will be able to develop informed targeted therapeutic strategies.

4.4.1 Pro-inflammatory cytokines. Increased production of inflammatory mediators can lead to tissue injury and organ failure [306]. It has been shown that mammals respond to endotoxins such as lipopolysaccharide (LPS) from Gram-negative bacteria and exotoxins such as toxic shock syndrome toxin 1 (TSST-1) from Gram-positive bacteria, by producing a variety of immune cell mediator such as TNF-α and IL-1β which have been shown to trigger lethal shock.

In our study, pro-inflammatory cytokines were elevated in septic patients when compared to healthy individuals. TNF-α, IL-1β, IFN-γ, IL-7 and IL-15 were modulated during sepsis, although the level of each cytokine varies from one patient to another. TNF-α was the only pro-inflammatory cytokine observed to be greatly increased in all four septic patients on the blot array with induction levels of 1738.5, 781.2, 875.8 and 955.9 for patient SE, SF, SG and SH respectively (Table 4-2). IFN-γ was significantly increased in two of the four patients on the cytokine blot array, patient SE with a 125.3-fold increase and patient SG with a 24.4-fold increase. In contrast, IL-1β was only elevated in patient SG by 736.7 fold while IL-7 and IL-15 were only elevated in patient SH with increases of 149.6- and 7.9-fold respectively.

4.4.1.1 Tumor necrosis factor-alpha (TNF-α). TNF-α was the only factor on the array found to be significantly up regulated in all four septic patients when compared to healthy individuals. It is often characterized as the prototype of a host damaging cytokine [307,308]. TNF-α has been shown to be one of the first cytokine secreted during sepsis. It is a potent activator of inflammation reactions of the innate immune system, including induction of cytokine production, activation and expression of adhesion molecules and growth stimulation [303,309]. Supporting the data on this cytokine array, previous studies have also demonstrated elevated pro-inflammatory cytokines, including TNF-α in septic patients and animal models of sepsis [300,303]. In addition it has also been demonstrated that infusion of TNF-α into animals induced characteristic septic symptoms while neutralizing TNF-α impaired the host defence against pathogens [307,310].

At low concentrations, TNF-α is involved in the maintenance of homeostasis of the immune system. It has many protective roles and biological functions as part of the physiological host defence against infections, trauma, or ischemia, including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation [311]. In contrast, excessive TNF-α production, as observed during sepsis leads to a harmful and often lethal systemic endotoxin like response which includes fever, hypotension, lactic acidosis, disseminated intravascular coagulation, shock, organ failure and death [312]. Multiple organ failure in patients with septic shock is often characterized by increased apoptosis throughout the body of lymphocytes and macrophages [166]. The apoptosis in septic shock appears to be triggered by signalling via members of the TNF-α receptor family [313], and induces activation of the Fas-associated death domain and caspase machinery [314]. The levels of TNF-α observed in the septic sera on our antibody array were extremely high when compared to the levels found in the healthy sera, and thus supports

the notion that extremely high levels of this cytokine activates apoptosis which plays a major role in tissue damage and organ failure during sepsis.

Massive TNF-α release can activate in turn other mediators of the inflammation cascade including higher expression levels of pro-inflammatory cytokines, anti-inflammatory cytokines and chemokines that all in concert participates in the pathogenesis of sepsis and septic shock [172]. On the antibody array, we demonstrated that other cytokines and chemokines such as IL-1β, IL-10, IL-6 and IL-8 are also modulated in the sera of septic patients which could be partly due to TNF-α's activation. Its commonality in all patients may imply that TNF-α is needed in a variety of responses to different infections. The other pro-inflammatory cytokines were differentially modulated between the different septic patients. IL-1β is modulated in patient SG, IFN-γ is modulated in patient SE and SG, while IL-7 and IL-15 are modulated in patient SH only.

4.4.1.2 Interleukin-1 beta (IL-1β). IL-1β, was induced in patient SG by 736.7-fold (Table 4-2). It has been shown to be induced by nearly all microbes and microbial products [315] and is an important mediator of the inflammatory response. IL-1β is produced by activated macrophages as a proprotein and is proteolytically processed to its active form by caspase 1 (CASP1/ICE). It is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. Also, similarly to TNF-α, IL-1β also increases the expression of other cytokines, such as TNF-α, IL-6 and IL-8. The increased production of other cytokines modulated by IL-1β exacerbates the uncontrolled systemic inflammation observed during sepsis.

In addition, IL-1β has been shown to activate neutrophils which induce a shock-like state in animal models [316]. In different models of local systemic inflammation, both in vitro and in

vivo, IL-1β acts synergistically with TNF-α [317] by increasing apoptosis and other mediators of inflammation. IL-1β, even though not greatly increased in all the serums of septic patients, might contribute synergistically with high levels of TNF-α, to increase the harmful effects observed during sepsis.

Increased plasma IL-1β has been reported in patients with various infections, in patients with sepsis and septic shock, as well as in healthy individuals injected with LPS [318]. Clinical trials using intravenous IL-1Ra, which was shown to block IL-1β activity were performed without reaching a statistically significant reduction in mortality even though in animal models of systemic inflammation (such as in septic shock), specific blockade of IL-1β with IL-1Ra resulted in a reduction in the severity of the inflammation [316,317]. These treatments were not effective and the reasons for this could be due to varying levels of IL-1β in different patients and also to the different pattern in cytokine expression in the sera as observed in our blot arrays. Patients with low IL-1β levels would not be expected to respond to a treatment using IL-Ra.

4.4.1.3 Interferon-gamma (IFN-γ). IFN-γ is a pro-inflammatory cytokine regulated by other cytokines such as TNF-α and IL-4 [319]. Elevated levels of IFN-γ were observed in the sera of septic patients SE (125.3-fold increase) and SG (24.4-fold increase) (Table 4-2). IFN-γ plays a complex and central role in the resistance of mammalian hosts to pathogens by activating several important antimicrobial effector functions in macrophage and neutrophil, including tumour cell cytotoxicity, antimicrobial activity, and the induction of specific MHC antigens, which increases receptor-mediated phagocytosis and pathogen killing [320]. IFN-γ also up-regulates the expression of chemokines and adhesion molecules which helps to direct leukocytes to sites of infection [321].

IFN-γ is an important immunomodulatory agent that is stimulated during infection and is of critical importance in the aid in host defence after the onset of sepsis. Nevertheless studies have shown that excessively high levels are deleterious [306,322]. IFN-γ antibody decreases bacterial load and attenuates mortality in various models of sepsis, thus implicating IFN-γ in the lethality of the disease [322,323]. In addition, mice lacking a functional IFN-γ response have been reported to have decreased natural resistance to infections [320,324]. These studies confirm that an impaired IFN-γ production is associated with worsened patient outcome during sepsis [320,325]. Lower levels of IFN-γ during sepsis can be detrimental by decreasing the clearing of bacteria and bacterial products which favors a greater uncontrolled infection. Increased production of IFN-γ can also be deleterious during sepsis by increasing the production of other inflammatory cytokines and the production of nitric oxide thus favoring the uncontrolled systemic inflammatory response [326].

4.4.1.4 Interleukin-7 (IL-7). When immune system is first challenged by exposure to antigen, naïve T cells become activated and undergo rounds of expansion as they differentiate into effector cells [327]. T lymphocytes play central roles in all adaptive immune responses against protein antigens by recognizing and killing cells infected with intracellular viruses and microbes [326]. T-cell population has been shown to be dependent on the presence of IL-7 for its persistence and survival, thus, IL-7 is now known as a central regulator of homeostasis for T lymphocytes [328]. Patient SH showed a 149.6-fold increase of IL-7 when compared to healthy individuals. Homeostasis of peripheral T cells through IL-7 is achieved by a combination of programmed or regulated cell death and responses to survival factors [327]. IL-7, while inducing the proliferation and differentiation of immature thymocytes, it stimulates the expressions of the

anti-apoptotic gene Bcl-2 in order to protect thymocytes from apoptosis [329]. Circulating levels of IL-7 increase in response to T-cell depletion suggests a role in T-cell regeneration [330].

In addition, IL-7 and IL-15 augment natural killer (NK) cell function [331] while certain precursor B-lymphocyte cell lines rapidly undergo apoptotic cell death following IL-7 withdrawal which could be detrimental during sepsis because mature B lymphocyte help secrete antibodies necessary to clear the infection [332].

4.4.1.5 Interleukin-15. IL-15, is involved in numerous processes, including recruitment and activation of T cells, maintenance of T cell memory, activation and proliferation of B cells and natural killer, activation of neutrophils, and inhibition of apoptosis [333]. We observed on the array an up-regulation by 7.9 fold in the serum of patient SH when compared to the levels in the serum of healthy individuals. IL-15, similarly to IL-7, is produced by macrophages in a wide variety of tissues after stimulation with LPS and in infections with various microorganisms in mice and humans [334].

IL-15 is a potent inhibitor of several apoptotic pathways in vitro on activated human T and B cells in response to various stimuli [314,335]. It inhibits apoptosis via the induction of the anti-apoptotic molecules Bcl-2 through activation of transcription factors STAT-5 and c-Myb [329,336]. IL-15 also inhibits TNF-α-mediated apoptosis by blocking the adaptor protein recruitment TRAF2 to the TNR1 [337]. Thus, IL-15 is a powerful apoptotic antagonist and can help prevent multiple organ failure caused by increased apoptosis observed during sepsis and septic shock [314]. This is supported by a study showing that IL-15 over-expression in mouse model can prevent TNF-α-induced apoptosis and protect against *E. coli*-induced shock [314].

Consequently, the up-regulation of this pro-inflammatory cytokine, suggest a protection against TNF-α mediated apoptosis which is greatly increased during sepsis, while a decreased expression of IL-15 during sepsis would increase the risk of organ failure due to increased apoptosis.

4.4.1.6 Interleukin-6 (IL-6). IL-6 is a pleiotropic cytokine with a wide range of biological activities that has been shown to possess both pro-inflammatory and anti-inflammatory properties. IL-6 was elevated by 146.0 and 1188.2 fold in septic patient SG and SH (Table 4-2) when compared to healthy individuals. Conventionally, IL-6 is considered to be a pro-inflammatory cytokine based on its pyrogenicity and its ability to induce a hepatic, acute-phase protein response. IL-6 also appears to down-regulate TNF-α expression with would categorize it as an anti-inflammatory cytokine [338].

IL-6 is synthesized by a wide range of cells including mononuclear phagocytes, monocytes/macrophages, vascular endothelial cell, fibroblasts, and T cells in response to endotoxin, IL-1β and TNF-α.[339] It has been demonstrated to regulate immune reactivity, the acute-phase response and inflammation which makes it also is a key mediator in multiple organ failure during sepsis [340]. IL-6 is the primary factor responsible for the induction of a broad array of acute phase proteins in response to systemic injury or inflammation [175] such as α-1-acid-glycoprotein or C-reactive protein. In contrast, IL-6 has also been demonstrated to induce direct *in vitro* myocardial depression in several models of sepsis, although not in others [175,341]. Thus, IL-6 appears to be both a marker and a mediator of sepsis [340].

Plasma IL-6 concentration has been directly correlated with risk of death due to sepsis [317,342], but it has been also demonstrated that the persistent elevations in IL-6 appears to be important

than initial or peak levels in terms of outcome during sepsis [340,343]. The up-regulation seen on the blot array further indicates IL-6 involvement during sepsis in a number of patients.

4.4.2 Anti-inflammatory cytokines.

During the later stage of sepsis, anti-inflammatory mediators are produced to counteract the pro-inflammatory effects induced during the early phase [311]. Anti-inflammatory cytokines can either impair the local antibacterial effectors mechanisms or can diminish systemic toxicity produced by bacteria depending on expressed levels [344]. In our cytokine blot array, three anti-inflammatory mediators were observed to be up-regulated in different septic patients. These anti-inflammatory cytokines include IL-10, IL-13 and IL-5.

4.4.2.1 Interleukin-10 (IL-10), Interleukin-13 (IL-13). IL-10 and IL-13 have pleiotropic effects in immunoregulation and inflammation. The expression of both cytokines is modulated by a variety of cell types, including T cells, monocytes/macrophages, dendritic cells, and epithelial cells. They are potent anti-inflammatory cytokines that suppresses macrophage inflammatory functions during sepsis which subsequently prevents the production of TNF-α, IL-1β, IFN-γ, IL-8 and IL-12 [302,345]. IL-10 and IL-13 have been observed to be up-regulated by 126.1- and 4.8-fold respectively in the serum of patient SH (Table 4-2).

IL-10 and IL-13 act as feedback regulator by initially being induced by the initial pro-inflammatory mediators, such as TNF-α and IL-1β and then subsequently, their endogenous production inhibits NF-κB production thus suppressing endogenous TNF-α, IL-1β and chemokine synthesis [344,346]. In addition, monocyte deactivation observed in sepsis is partially due to IL-10, while augmented substrate adherence of monocyte due to IL-13 [347]. Furthermore, both IL-10 and IL-13 enhance B cell survival, proliferation, and antibody

production. In addition, IL-10 also induces neutrophils apoptosis during the resolution of inflammation [348].

During sepsis, the administration of anti-IL-10 antibodies can increases endotoxin and bacteria-induced mortality in mice, whereas the systemic injection of IL-10 have been shown to prevent septic shock in animals by inhibiting the synthesis of TNF-α, IL-1β, IL-6, IL-8, IFN-γ and others [349]. Also, blocking IL-13 was detrimental to survival after cecal ligation and puncture (CLP), further indicating that it also serves as a protective cytokine during the evolution of sepsis [350].

While increased levels of IL-10 and IL-13 have been shown to be beneficial and vital for survival during sepsis by contributing to the immunosuppressive effects [300], it can also, in other cases, be detrimental by enhancing the inflammatory response. Studies also reported that the highest level of IL-10 relative to those of TNF-α was associated with poor outcome in sepsis patients [347,351].

4.4.2.2 Interleukin-5 (IL-5). IL-5 enhanced B cell survival, proliferation and antibody production is known to enhance humoral immunity and inhibit cell-mediated immunity, which result in protective effect for pathogens removed primarily through humoral mechanisms [352]. Studies have also shown that IL-5 is augmented significantly following anti-parasite therapy treated patients [353]. Although there are very few studies that directly link IL-5 with sepsis, we found that this cytokine was significantly up-regulated in the serum of patients SG and SH by 27.8- and 19.2-fold respectively. This suggests that this cytokine might be linked the anti-inflammatory processes during sepsis and septic shock. Further studies are needed to determine the precise role of IL-5 during this disease.

4.4.5 Chemokines. Chemokines are small chemotactic cytokines involved in immunoregulation and inflammation processes. They are a large superfamily of structurally and functionally related molecules that induce directional migration and activation of specific leukocyte population from the vasculature into the tissue during inflammation [354]. Chemokines are 70-90 amino acids in length and are divides into four subfamilies, C, CC, CSC and CXC, based on their conserved cysteine residues located near the amino terminus relative position of their cysteine residues [354].

There are at least 50 distinct chemokines, which participate in recruiting different leukocyte population to the site of inflammation [354]. CXC chemokines, which include GRO and IL-8, are potent chemoattractants for neutrophils, whereas the CC chemokines, including MCP-1, and MIP-1, can attract monocytes, lymphocytes, basophils, eosinophils and natural killer cells [355].

In this study, we measured elevated levels of many chemokines in three of the septic patients. It was previously shown that many chemokines are up-regulated simultaneously upon LPS-stimulation which is consistent with our result [356]. Also, this elevation indicates that a wide variety of chemokines may orchestrate under pathophysiological conditions and play a role in inflammatory reactions [356]. In addition, another study measured extremely high levels of all chemokines in the serum during acute-phase. The levels were also shown to be significantly higher in non survivors compared with survivors and in patients with septic shock compared with patients with sepsis [355]. In this study we observed an up-regulation of chemokines with fold increase that range from 2.4- to 13.2-fold which further emphasize the importance of chemokines in recruiting leukocytes during the course of sepsis.

4.4.5.1 Interleukin-8 (IL-8). One of the chemokines expressed on the array, IL-8, also known as CXCL8, is a chemotactic factor for neutrophils, basophils, and T-cells during the inflammatory response [357]. Chemokine levels, including MCP-1 and IL-8 have been correlated with LPS plasma level, which further suggests their important role during sepsis [358]. IL-8 was shown to be undetectable in the serum of our healthy volunteers while it was elevated in two of the septic patients, SF and SH, by 9.1- and 34.4-fold correspondingly.

IL-8 is released from several cell types in response to multiple inflammatory stimuli including pro-inflammatory cytokines such as TNF-α and IL-1β [357,359]. During inflammation, IL-8 levels will sustain for a prolong period of time when compared to other cytokine which are typically produced and cleared in a matter of a few hours [357]. IL-8 will initially mediate the migration of leukocytes across the vascular endothelium [359]. Once leukocytes are present in the tissue, neutrophils are activated by IL-8 to produce oxygen free radicals, release proteases and begin to phagocytose and kill ingested pathogens [360]. IL-8 will also inhibit neutrophil adhesion to cytokine-activated endothelial cells, which protects these cells from neutrophil-mediated damage [359].

Although IL-8 attract neutrophils during local inflammation in order to increase the inflammation process, during sepsis, increased levels of IL-8 are found in the plasma which will produce a gradient away rather than in the direction of the initial inflammation [356,357]. This anti-inflammatory role caused by the recruitment of leukocyte in the plasma versus the local infection makes clearing of the infection a greater challenge during sepsis. The opposed gradient will reduce recruitment of neutrophils at the site of inflammation thus limiting the effect of the pro-inflammatory mediators released by these cells. This is supported by a study where injection

of IL-8 in rabbits and rats significantly reduced *in vivo* neutrophil migration towards inflammatory foci [361].

This chemokine has been shown to be significantly higher during multiple organ failure, in non survivors compared with survivors and in patients with septic shock compared with patients with sepsis [355,362]. It is rapidly synthesized during inflammation where it acts to recruit and activate acute inflammatory cells [357].

4.4.5.2 Monocyte chemo-attractant protein 1 (MCP-1/CCL2) and Monocyte chemo-attractant protein 2 (MCP-2/CCL8). MCP-1 has a chemotactic activity for monocytes and basophils, while MCP-2 has a chemotactic activity for monocytes, lymphocytes, basophils and eosinophils. Both chemokines are expressed in multiple cell types, including fibroblasts, endothelial cells, monocytes, and macrophages following stimulation with TNF-α, IL-1β, IFN-γ or endotoxin, which are all involved in the pathogenesis of sepsis [356,363,364]. We observed increased levels of MCP-1 in the serum of patients SF, SG and SH by 7.2-, 13.2- and 17.9-fold respectively, while MCP-2 was increase in patient SH by 4.3-fold (Table 4-2).

Mice challenged with Gram-negative bacteria which were administered MCP-1 have showed a diminished lethality to endotoxemia [365]. This indicates that the increased levels of MCP-1 and possibly MCP-2 found in septic patients may furthermore constitute a host compensatory response to reduce the deleterious effects of sepsis development [364]. In contrast to MCP-1, MCP-2 did not show an increase in patients with Gram-negative infections compared with controls, although it was showed to be increased in almost all Gram-positive infections [364]. Consistent with our results, MCP-1 when expressed exerts a protective role during sepsis triggered by both Gram-positive and Gram-negative bacteria, by eliciting and recruiting various

leukocyte subpopulations required to locally contain the primary traumatic insult and eliminate microbes [363]. In addition, MCP-2 was only shown to be up-regulated in the only patient who survived with a Gram-positive infection, further suggesting an added beneficial effect due to MCP-2 expression in Gram-positive infections.

In contrast, other studies also showed that extremely high levels of MCP-1 and MCP-2 in the sera of patients with sepsis correlated with the more severe forms of sepsis and also was significantly higher in non survivors compared to survivors during sepsis and septic shock [355,358,364]. Blocking MCP-1 significantly decreased the survival rate in animal model of sepsis because of increased viability of bacteria due to the reduction in the recruitment and activation of leukocytes [363]. This implicates MCP-1 and perhaps MCP-2 in the pathogenesis of sepsis. These studies support the notion that a well-regulated expression of MCP-1 and MCP-2 levels is beneficial to the host, while overexpression or under expression might be detrimental. The up-regulation observed on our antibody array further supports previous studies showing an up-regulation of these chemokines during sepsis and septic shock.

In addition, anti-TNF-α treatment during endotoxemia caused inability of the myocardium to block IL-6 and MCP-1 expression which suggests another possible mechanism for the failure of anti-TNF-α therapies in the treatment of endotoxin shock and further emphasizes the complex nature of this multifaceted disease.

Growth-related oncogene alpha (GRO-α/CXCL1), Growth-related oncogene beta (GRO-β/CXCL2), Growth-related oncogene gamma (GRO-γ/CXCL3). GRO-α, -β, and -γ are CXC chemokines which are a family of chemokines that are specialized in monocyte arrest and that have neutrophil-activating and chemoattractant properties similar to those of IL-8 [366] GRO-α

was increased in the serum of patient SH by 5.4-fold while GRO β, and γ was observed to be increased in the serum of patients SF, SG and SH by 10.8-, 2.9- and 22.0-fold respectively.

GRO-α, β, and γ all act, through the CXCR2 receptor, as arrest chemokines for monocyte adhesion in various models of inflammation although they are unable to induce monocyte chemotaxis [366]. In addition, platelet activation leads to the release of GRO-α, which then attracts leukocytes and further activates other platelets [367]. This function provides a rapid essential protection against bleeding and in addition, catalyzes the slower formation of stable blood clots through the coagulation cascade [367].

GRO α, GRO β (MIP-2α), and GRO γ (MIP-2β) have shown to be expressed in response to a variety of inflammatory mediators, such as IL-1β, TNF-α, IFN-γ and LPS, in monocytes, fibroblasts, melanocytes and epithelial cells [356]. During sepsis, it was reported that an altered chemotaxis of neutrophils to GRO family was associated with a decreased expression of CXCR2 on various cells [368]. Thus during sepsis, the decrease of neutrophils to site of infection might be due indirectly to the GRO family since the receptor is decreased.

Accordingly, the elevated expression of GRO-α, GRO-β and GRO-γ in the serum of septic patients might be linked to a decreased expression of CXCR2 and to increased levels of others cytokines secreted during inflammation such as TNF-α, IL-1β, IFN-γ during sepsis.

4.4.5.3 Thymus and activation-regulated chemokines (TARC/CCL17). TARC (CCL17) is a CC chemokines with chemotactic activity for T lymphocytes, but not monocytes or granulocytes (136). TARC's receptor, CCR4 is highly expressed on Th2 cells and thus TARC is considered to by a key mediator in recruiting these cells [369,370]. The blot array showed that TARC was elevated by 5.8-fold in patient SF which further indicates the important role of chemokines

during sepsis. In addition, this chemokine plays an important role in T cell development in the thymus as well as in trafficking and activation of mature T cells.

During sepsis, coagulation abnormalities are common [371]. Platelet aggregation studied during sepsis give conflicting results. Studies have shown an increased platelet aggregation [372] while other studies have shown an inhibited platelet aggregation [373]. TARC, which is produced during inflammatory responses, coupled with low levels of ADP or thrombin, can serve as strong rapid activators of platelet aggregation and adhesion after its binding to CCR4 [374]. TARC consequently stimulates platelet shape change, aggregation, and adhesion to collagen or fibrinogen in addition to attracting T lymphocytes and B lymphocytes [374,375].

TARC gene expression was shown to be up-regulated simultaneously with many other chemokines upon LPS-stimulation [356,376]. TARC mediates selective migration of Th2 cells into inflamed tissues. In addition, CCR4 has been shown to play a key role in models of sepsis in that CCR4 deficient mice exhibited significantly decreased mortality on administration of high or low dose bacterial LPS compared with mice with the CCR4 receptor suggesting a protective role for TARC and its receptor in sepsis [377].

In addition the similar roles of TARC and GRO family chemokines during sepsis further indicates that there are overlapping and redundant roles between chemokines during the disease.

4.4.5.4 Stromal cell-derived factor-1 (SDF-1/CXCL12). SDF-1 is a chemokine with chemotactic role for T lymphocytes, monocytes, and neutrophils, which all express CXCR4, the only known receptor for SDF-1 [354]. SDF-1 and CXCR4 together play crucial roles in many biological processes including hematopoiesis, cardiogenesis, vasculogenesis, neuronal development and immune cell trafficking [378]. On our blot array, patient SH had an increase of SDF-1 expression

in the serum by 2.1-fold (Table 4-2) when compared to healthy individuals. Stromal cell-derived factor (SDF-1) activates leukocytes in response to pro-inflammatory stimuli such as LPS or TNF-α. Thus, inhibiting SDF-1 or its receptor CXCR4 disrupts normal immunological response by deregulated lymphocyte trafficking.

The response of Toll-like receptor 4 (TLR4) to LPS is thought vital for resisting infection while aberrant TLR4 signalling may initiate inflammatory conditions including sepsis syndrome [379]. It was observed that TLR4 signalling is enhanced when CXCR4 is blocked and suppressed TLR4 signalling occurs with an increased expression of CXCR4 [379]. Therefore, CXCR4 exert local control of TLR4 and this suggests that SDF-1/CXCR4 expression suppresses TLR4 signalling and lead to increased infection.

High levels of expression of IL-10, TNF-α and IFN-γ down regulate CXCR4 expression, thus indirectly reducing SDF-1 expression [350]. In addition, other studies have also shown that the expression of SDF-1 is unaltered by certain inflammatory stimuli [381] suggesting that the expression of SDF-1 might be dependent on the infecting organism.

4.4.5.5 Monokine induced by gamma-interferon (MIG/CXCL9). MIG was up-regulated in patient SH by 3.8-fold when compared to healthy individuals. Its function has not been specifically defined; however, unlike many CXC chemokines, MIG is thought to be involved only in T cell trafficking. MIG does not share receptors with any other chemokines and is recognized only by CXCR3 receptor that has only been found on T lymphocytes. Thus it is thought that MIG is selective for activated T-lymphocytes and it may be inferred that the states of viral infection may be regulated via MIG receptor expression [382].

IFN-γ stimulated mononuclear cells have been shown to induce a sustained expression of Monokine induced by interferon-γ (MIG/CXCL9) which then attracts activated T-cells through its chemokine receptor CXCR3 [381,382]. Also, MIG, similarly to SDF-1, was showed to be enhanced during the early and late stages of infection in cardiac tissues of animals infected with *Tripanosoma cruzi* [381].

However it was shown that targeting MIG alone does not directly modulate the inflammatory response within the heart. This suggests that other inflammatory mediators are able to regulate inflammation in this tissue [383].

4.4.5.6 Macrophage inflammatory protein 5 (MIP-1δ/CCL15). MIP-1δ (CCL15) is a CC chemokines that attract T cells, monocytes, neutrophils and eosinophils [384]. MIP-1δ binds to receptors CCR1 and CCR3 on various cells inducing signalling which can alter the intracellular calcium concentration in these cells [385]. This chemokine was increased in patient SG by 2.4-fold (Table 4-2).

Activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB) (through c-Jun N-terminal kinase (JNK)/mitogen activated protein kinase (MAPK) and MAPK extracellular kinase (MEK) pathways) regulates the transcription of MIP-1δ in monocytoid cells [384]. MIP-1δ has been shown to be expressed in response to oxidative stress [384] and in addition, neutrophils in the plasma release CCL15 proteolytically in order to induce monocyte infiltration into sites of inflammation [368].

The expression of MIP-1δ during sepsis has not been extensively studied and thus its exact role during the process of this disease is unknown. We observed an elevated level of this chemokine in patient SG.

4.4.6 Others.

Other factors included on the blot array cannot be classified under the pro-inflammatory, the anti-inflammatory or the chemokines categories. These factors which are growth factors, angiogenic factors, and hormones possess many characteristic of cytokines. These factors specifically are leptin, epidermal growth factor (EGF), angiogenin, and platelet derived growth factor-BB (PDGF-BB). These factors were elevated in the septic sera, with the exception of angiogenin which showed a down-regulation, in the different septic patients.

4.4.6.1 Leptin. Leptin, originally described as an antiobesity hormone, was found after extensive research, to be a crucial cytokine during inflammation and sepsis [387]. Leptin was shown to modulate monocyte-macrophage function and to regulate pro-inflammatory response [388]. On our blot array we observed an increased serum leptin level in patient SE (8.0-fold increase) and patient SH (8.4-fold increase) when compared to the healthy individuals (Table 4-2).

It was shown that TNF-α and IL-1β are both potent inducers of leptin in mouse macrophages, which results in an enhanced cytokine production, including IL-6, IL-1β and TNF-α [388]. Other studies in relation to leptin showed that both TNF-α and leptin depress ventricular myocyte shortening (inhibition of cardiac myocyte contraction), which is likely mediated through a nitric oxide (NO)-dependent mechanism [389]. In addition, during the impaired myocyte contraction, leptin and TNF-α showed no synergistic or additive effect between the two mediators [389]. Since increased leptin concentration has been reported during sepsis [389], this could imply that leptin may induce sepsis dependent myocardial dysfunction by depressing myocyte contraction.

4.4.6.2 Epidermal growth factor (EGF). Epidermal growth factor (EGF) has a profound effect on the differentiation of specific cells *in vivo*. It is a potent mitogenic factor for a variety of cultured cells of both ectodermal and mesodermal origin. EGF is known to activate epithelial cell migration and proliferation and in addition, accelerates wound and ulcer healing *in vivo* and *in vitro* [390]. In addition, EGF was shown to reduce bacterial translocation and blood infection when administered to rats receiving total parenteral nutrition [391]. Studies have not been performed to determine the role of EGF during sepsis. Here, we have shown that EGF levels are elevated in patient SE and SF by 7.9- and 7.3-fold respectively.

4.4.6.3 Angiogenin. Angiogenin a heparin-binding 14 kDa plasma protein with angiogenic and ribonucleolytic activity and is part of a family of endogenous antimicrobial proteins [392]. Interestingly, it was observed to be down-regulated in the serum of septic patient SE (2.2 fold decrease) when compared to the serum of healthy individuals. Angiogenin has been shown to induce a wide range of cellular responses necessary for angiogenesis by interacting with endothelial cells which including migration, proliferation, and tube formation [393]. In addition, human angiogenin, which can be induced during inflammation, exhibits microbicidal activity against systemic bacterial and fungal pathogens. The activity observed during inflammation suggests that angiogenin might contribute to systemic responses to infection [392]. The down-regulation observed in septic patients further suggests that angiogenin has important microbicidal activity. Lower microbicidal activity during sepsis leads to an increase bacterial load in the blood and to a less favourable outcome.

4.4.6.4 Platelet derived growth factor-BB (PDGF BB). Platelet derived growth factor-BB (PDGF BB) was observed to be increased by 7.1-fold in patient SF. PDGF BB is a member of the platelet-derived growth factor family and affects the process of normal wound healing. Shortly

after wounding, platelets within the fibrin plug releases PDGF-BB. Thereafter, PDGF-BB amplifies fibroblast expression and activates epithelial cell migration and proliferation, which are involved in wound healing [390].

The efficacy of PDGF is shown in various clinical trials involving pressure, neuropathic ulcer healing, and surgical wounds [394]. A preliminary study suggests that Becaplermin (PDGF-BB), the only growth factor approved by the Food and Drug Administration accelerates healing of separated surgical wounds [395].

PDGF BB, similarly to TARC, plays an important role in wound healing. Thus, further research is needed to investigate the role between sepsis and wound healing growth factors.

4.5 Conclusion

Various microbes and microbial products initiate host cellular signalling pathways that produce a surge of pro-inflammatory mediators. This is followed by chemokine production which regulates migration of cells and by an anti-inflammatory response to attenuate the initial response. In order to resolve the infection, the balance between the pro-inflammatory, the anti-inflammatory and the chemokine response must be tightly regulated. An imbalance or a deregulation of one or multiple factors of the response can lead, through multiple pathways, to cellular dysfunction, tissue injury, organ dysfunction, multiple organ failure and possibly death.

Interestingly, the only cytokine that was consistently elevated in the serum of septic patients when compared to normal is the pro-inflammatory cytokine TNF-α which further highlights its importance in the various pathways that are deregulated during the disease. Although the expression of the factors during sepsis varies from one patient to the other, our data indicate that

multiple key cytokines, chemokines and growth factors are expressed. Targeting a single factor during sepsis is insufficient in re-establishing a balanced and beneficial inflammatory response due to the multiple and overlapping cascades activated during the disease. We believe that individual and specific therapies will be developed by profiling the cytokine, chemokine and growth factor pattern of each patient.

It is worth noting that investigating the change in profile of these pro and anti-inflammatory mediators would be beneficial in identifying patterns of expression that may be used to predict progression of sepsis. Temporal changes in these mediators could also be used to identify synergistic combinations that would be associated with poor outcome or even patterns of cytokines that may lead to sustained immunosuppression that is experienced by many who survive sepsis. Identifying sepsis biomarkers in the sera of septic patients would increase our knowledge of the pathophysiological process occurring and would allow for a better targeted approach in treating the many morbidities.

Thesis Conclusion

This study characterized the inflammatory mediators from two different inducers of sepsis. The first inducer was pandemic H1N1 2009 Influenza A. This represented viral sepsis. The lung tissue from these samples demonstrated tissue injury, infiltration of immune cells and apoptotic cells in situ. The sera from pandemic H1N1 infected individuals contained high levels of the pro-inflammatory cytokines TNF-α, IL-1β and IFN-γ. These sera also demonstrated the ability to induce apoptosis in cultured fibroblasts which require STAT1 to mediate the apoptotic signal. The second inducer of sepsis was from bacteria. Similar to viral samples the bacterial septic sera samples had increased levels of TNF-α, IL-1β and IFN-γ. Characterization of these sera demonstrated the presence of other inflammatory mediators including chemokines. Different profiles of inflammatory mediators are induced by different classes of microbes. Characterization of these mediators and their temporal expression will better aid in the understanding of the complex nature of sepsis and in the development of better treatment strategies.

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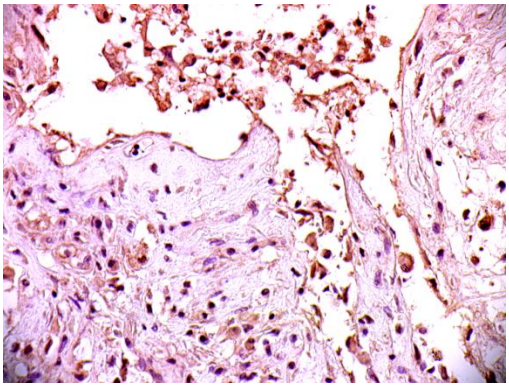
Appendix

Table A-1. Summary of immunohistochemical staining for H1N1 viral proteins and cytokines in remaining lung tissue.

Sample no.	IHC Score for virus	Location of staining	Cell types with positive staining for virus	TNF-α	IFN-γ	IL-1β
SJ RI-496-09	+	B/I	Airway Epithelium and Macrophages	+	++	Negative
SJ RI-497-09	+	C/I	Macrophages	++	+	Negative
HA AU-09-000110	Negative			Negative	Negative	Negative
HA AU009-000073	+	C/A/I	Alveolar Epithelial cells and Macrophages	+	+	Negative
AF-09-121	+	C/I	Macrophages	+	+	Negative
AF-09-174	+++	B/A	Type I and II pneumocytes	+	+	Negative
AF-09-195	+	C/I	Macrophages	+	Negative	Negative
AF-09-201	++++	B/A	Type I and II pneumocytes and epithelial cells	+	+	+
AF-09-206	+	B/A/I	Alveolar and glandular epithelium and macrophage	+	+	Negative
AF-09-216	+	B/A	Airway and alveolar epithelial	+	Negative	Negative
AF-09-234	+	C/I	Macrophage	+	+	Negative

Immunohistochemistry staining for viral nucleoprotein and cytokines TNFα, IL-1β and IFN-γ were performed on 5μm sections of lung tissue from autopsy samples. Proteins were detected by diaminobenzidine and counterstained with hematoxylin. The level of staining is indicated by the symbol + weak staining, ++ strong staining and +++ for heavy staining. Location of staining for viral protein A= alveolar, I= interstitial, C= cytoplasmic, N= nuclear and B= both

A



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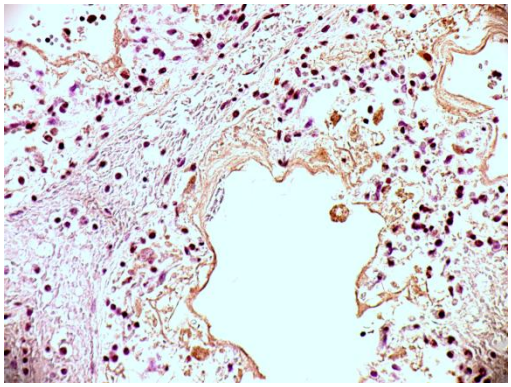

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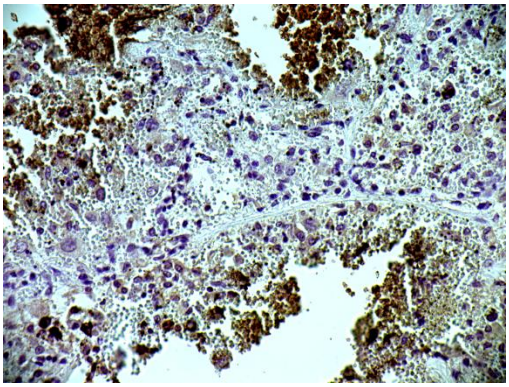

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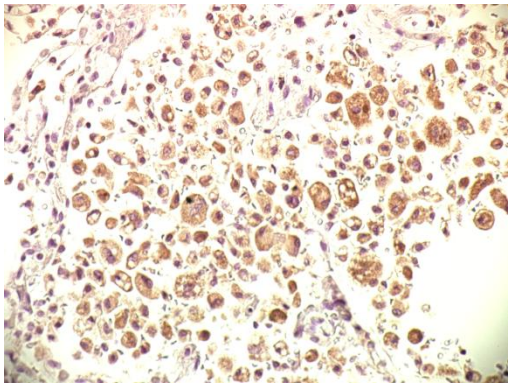

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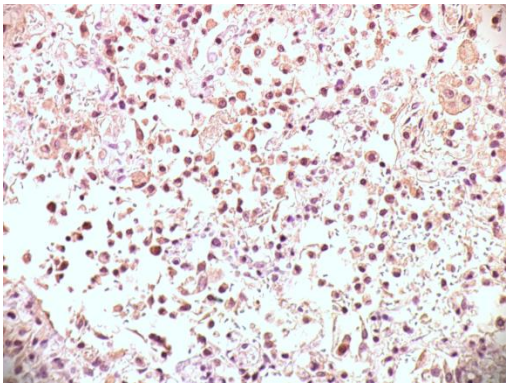

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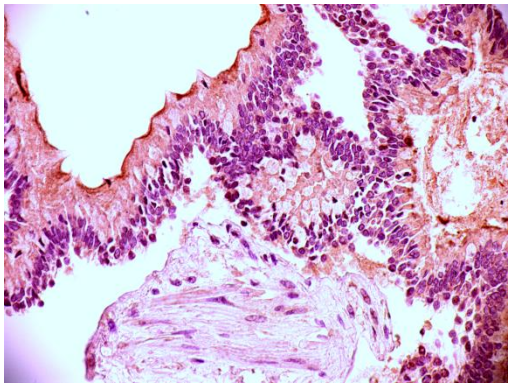

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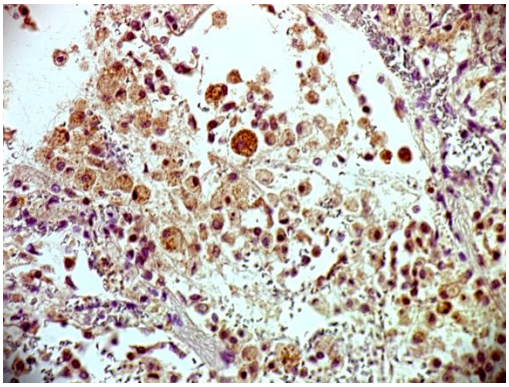

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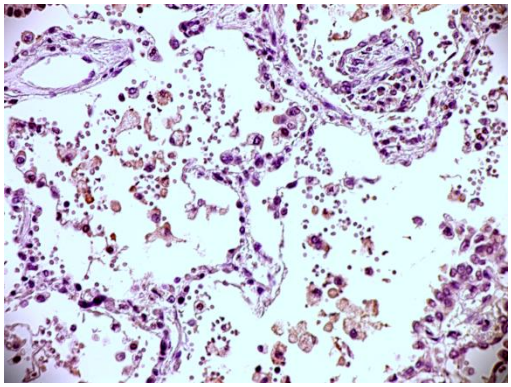

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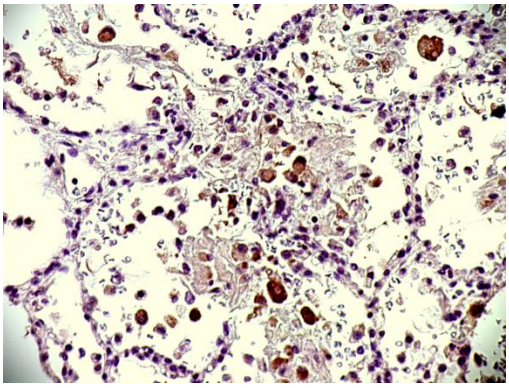

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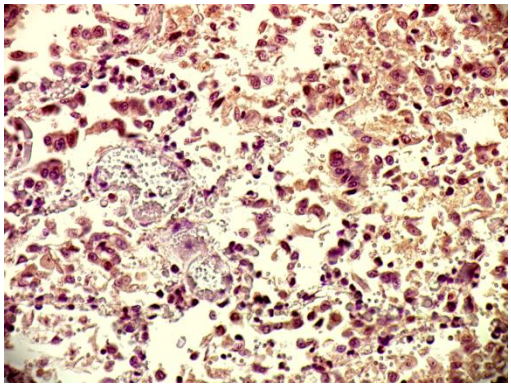

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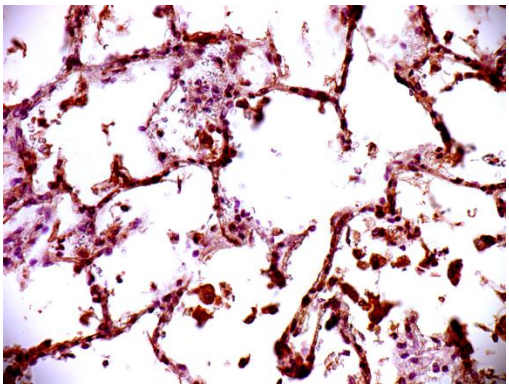

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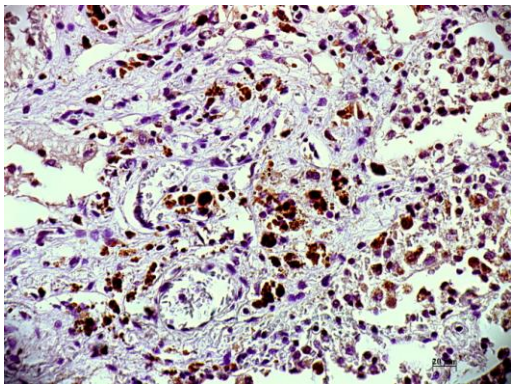

Figure A-1. TNF-α stained H1N1 infected lung sections. 5μm sections of formalin fixed paraffin embedded lung tissue from autopsy cases of H1N1 infection (A-K) and bacterial sepsis control (L).

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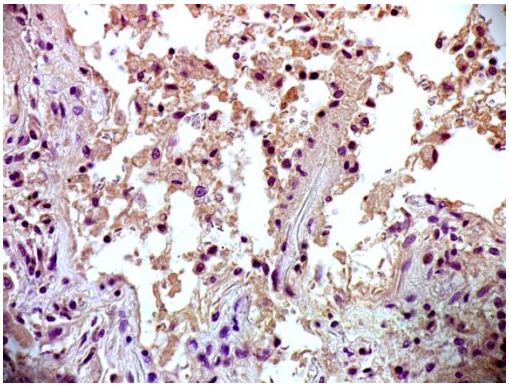

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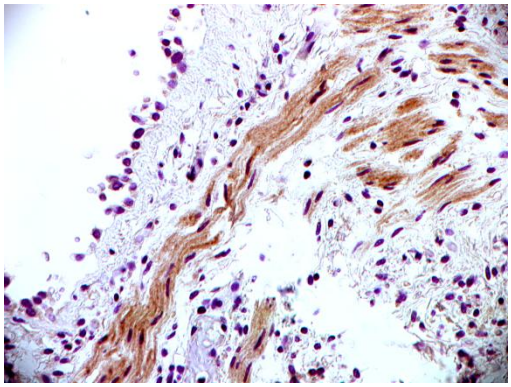

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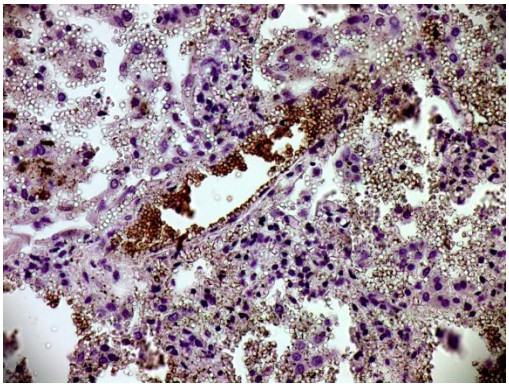

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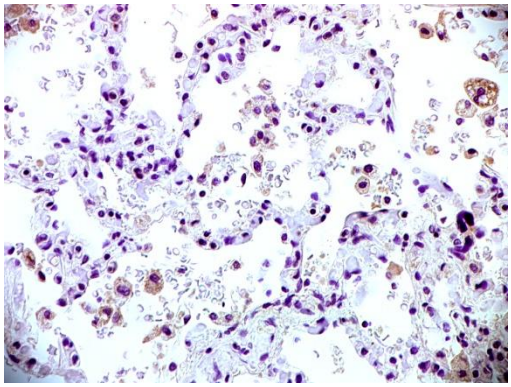

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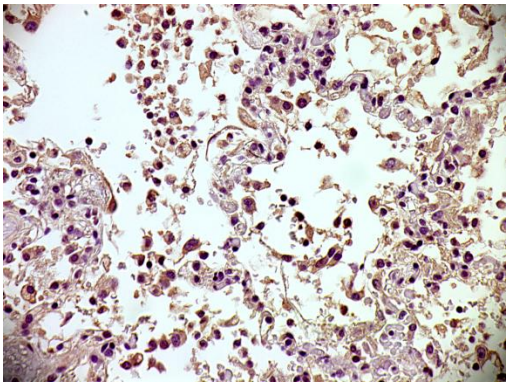

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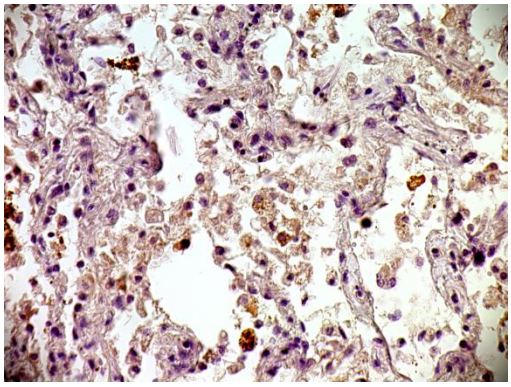

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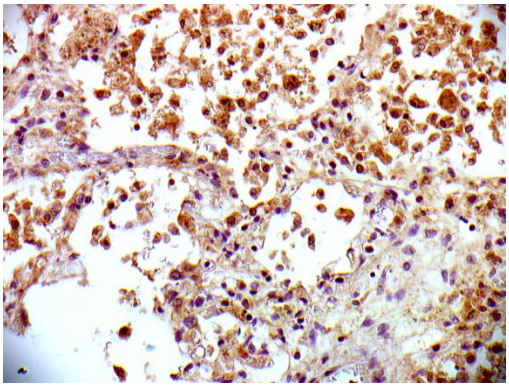

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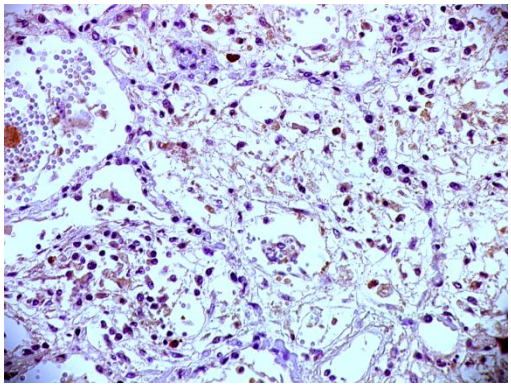

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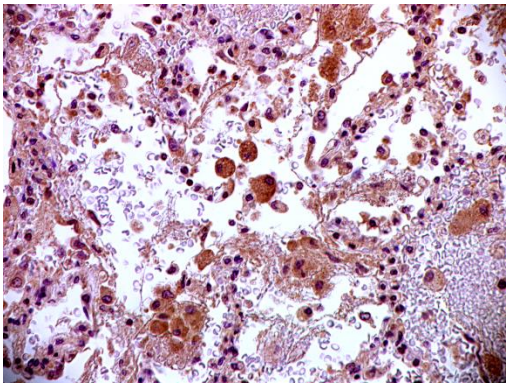

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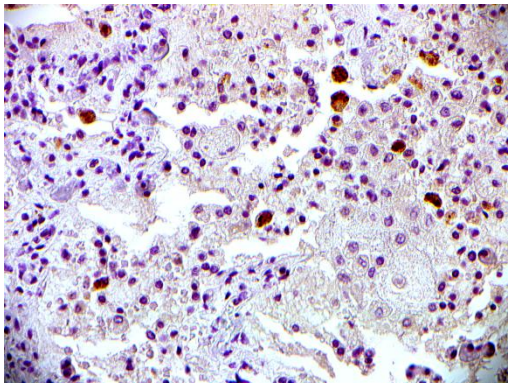

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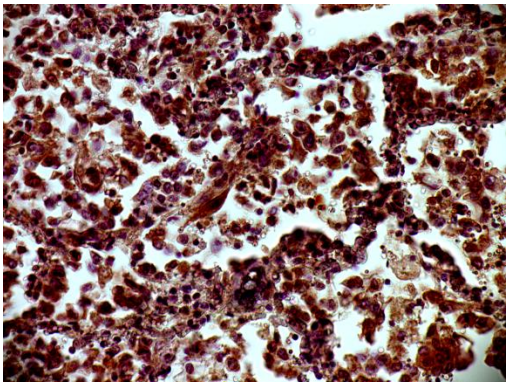

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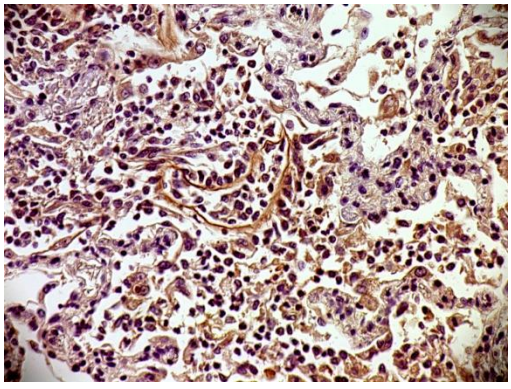

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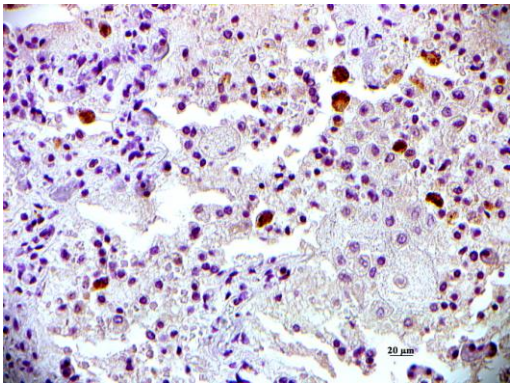

Figure A-2. IFN-γ stained H1N1 infected lung sections. 5µm sections of formalin fixed paraffin embedded lung tissue from autopsy cases of H1N1 infection (A-L) and bacterial sepsis control (M).

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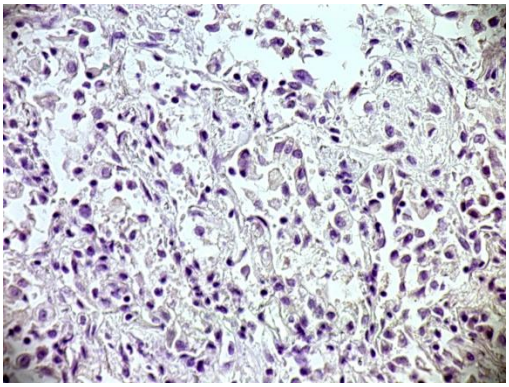

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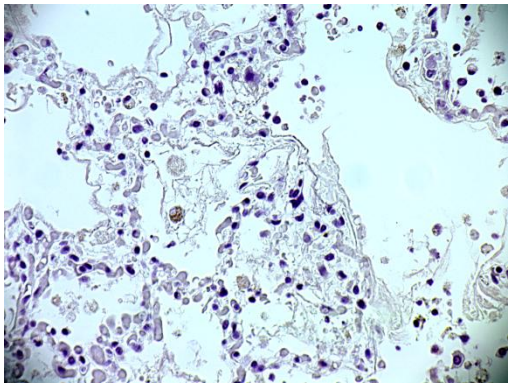

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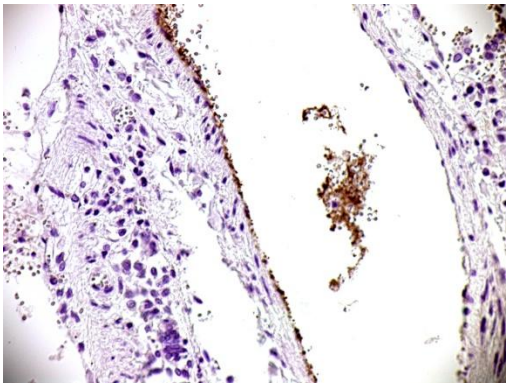

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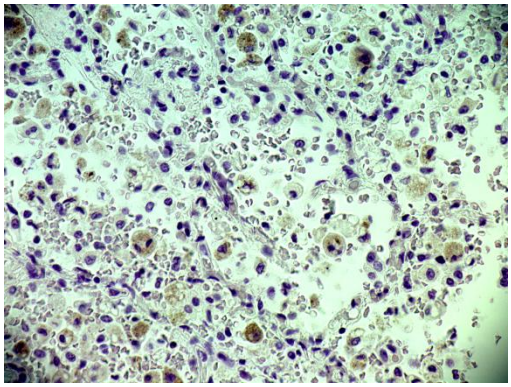

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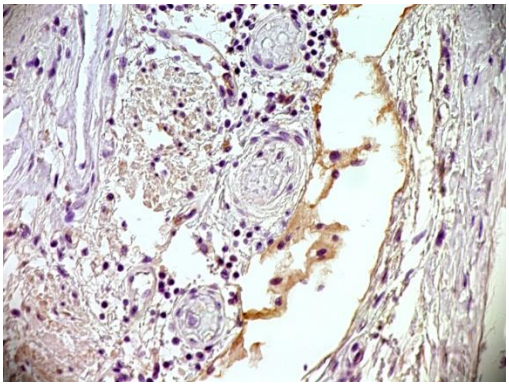

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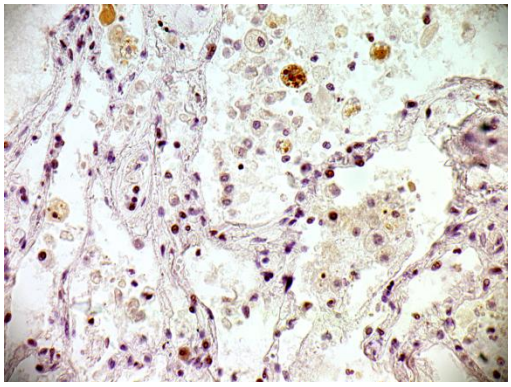

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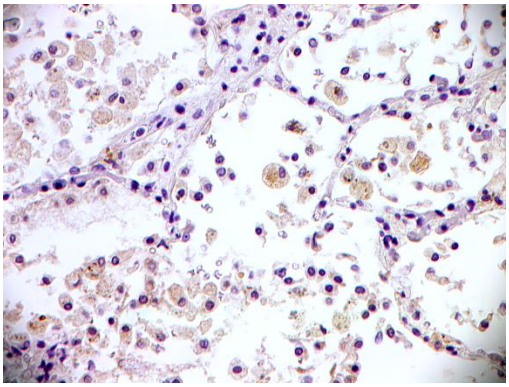

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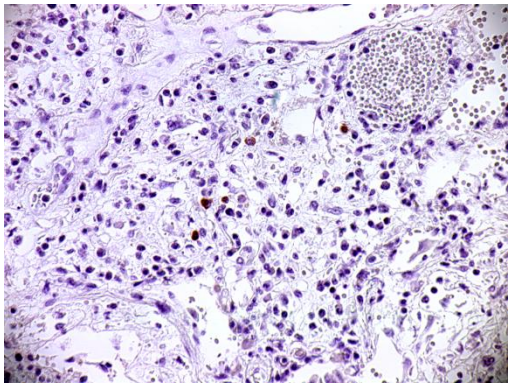

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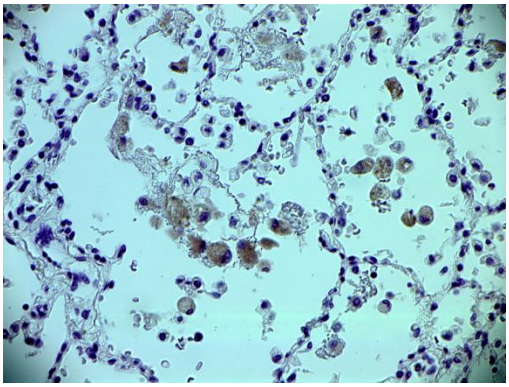

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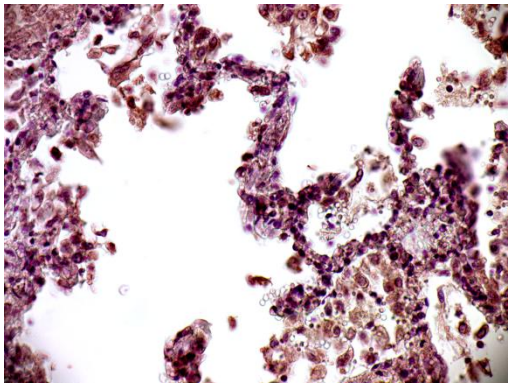

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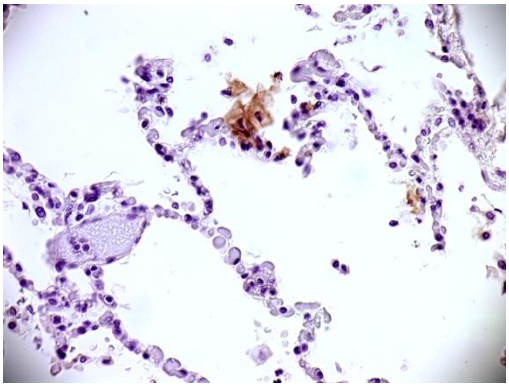

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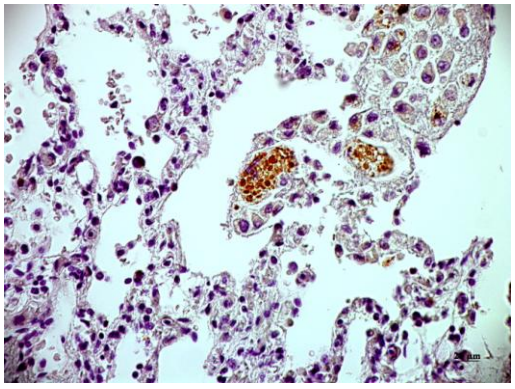

Figure A-3. IL-1β stained H1N1 infected lung sections. 5μm sections of formalin fixed paraffin embedded lung tissue from autopsy cases of H1N1 infection (A-K) and bacterial sepsis control (L).

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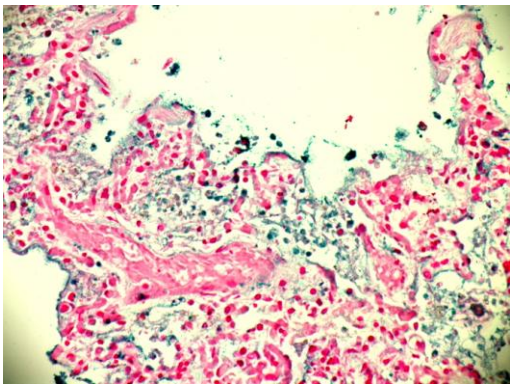

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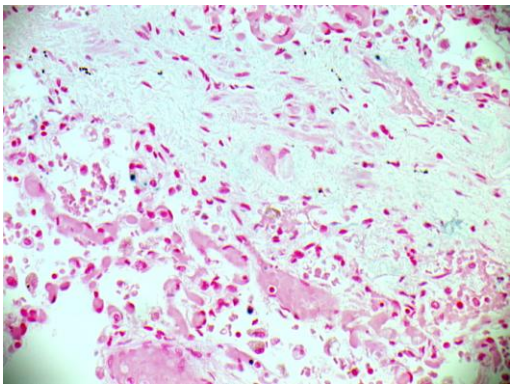

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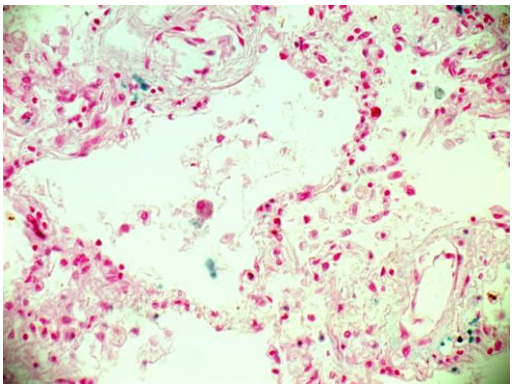

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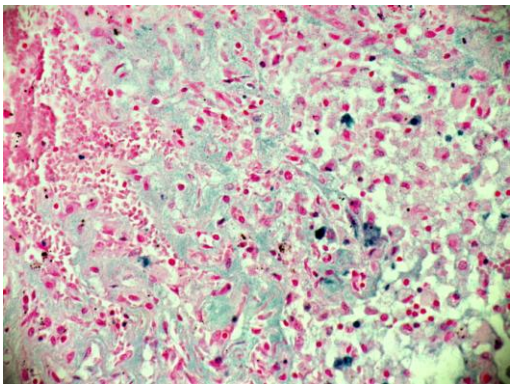

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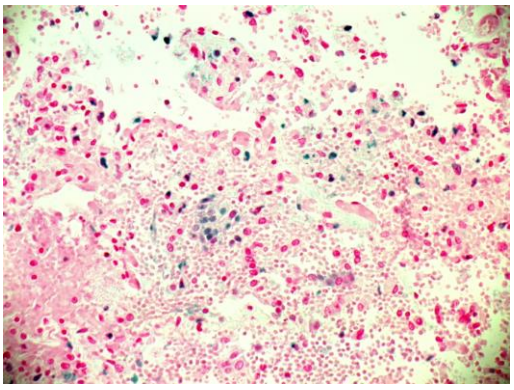

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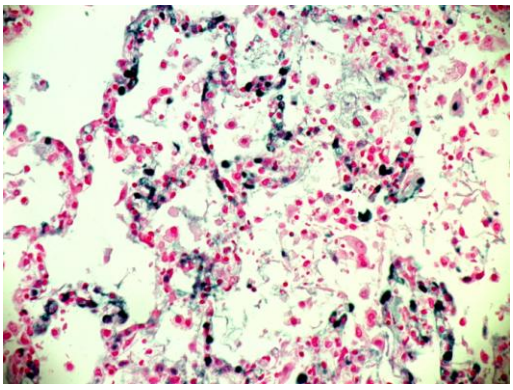

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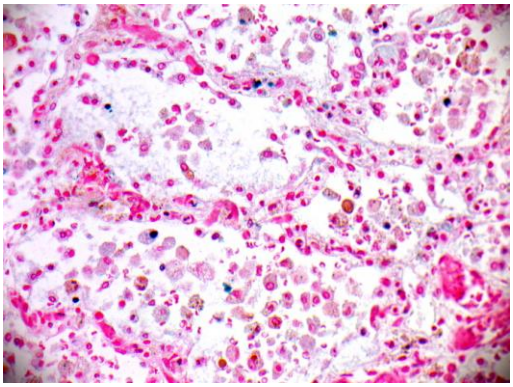

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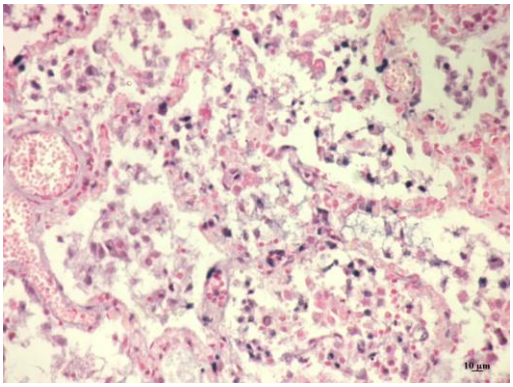

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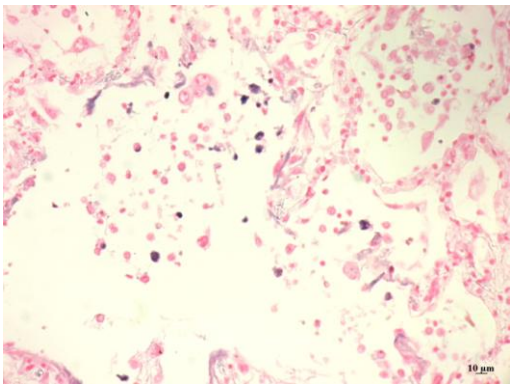

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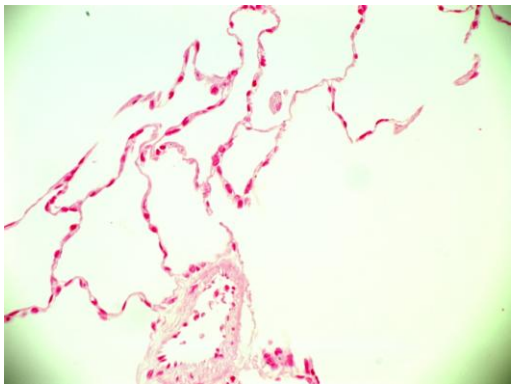


Figure A-4 Tunel assay on H1N1 infected lung tissue. Tunel staining was performed on 5 µm formalin fixed paraffin embedded H1N1 infected tissue (A-I) and uninfected lung tissue (J). Tunel positive cells stain blue. Tissues were counterstained with Nuclear Fast Red.

Table A-2. Trypan exclusion assay for uninfected and H1N1 infected sera on cultured fibroblasts.

	Cell Lines			
Sera	HT1080	2fTGH	U3A	U3AR
N1	0.8	0.7	0.6	0.9
N2	1	1.1	0.8	0.7
N3	0.6	1.1	0.7	0.9
N4	1.2	1	1.1	1.3
N5	0.9	1.2	1	0.7
N6	1.1	0.8	0.9	1
N7	1.2	1.4	1.2	0.8
N8	0.7	1	0.9	0.7
H1	23.2	22.1	3.5	21.5
H2	22.1	20.6	2.1	21.3
H3	18.5	19.4	2.8	18.3
H4	26.4	24.6	3.2	22.7
H5	28.4	25.9	1.8	25
H6	24	26.3	2.6	27.6
H7	23.9	23.2	3.2	21.8
H8	20.7	22.5	3.1	23.5

Trypan blue exclusion assay for 8 uninfected sera (N) and 8 H1N1 infected sera (H). Fibroblast cell lines were seeded at $5X10^4$ cells/well in a 96 well plate and were treated with either 5% uninfected sera or 5% H1N1 infected sera for 12 hours before the trypan blue assay. Values are averages for percent blue staining cells for each serum.

Table A-3. TUNEL assay for 8 uninfected and 8 H1N1 infected sera treated cultured fibroblasts.

	Cell Lines			
Sera	HT1080	2fTGH	U3A	U3AR
N1	0.1	0.08	0.07	0.07
N2	0.12	0.1	0.09	0.09
N3	0.08	0.1	0.13	0.08
N4	0.12	0.07	0.09	0.11
N5	0.09	0.07	0.12	0.06
N6	0.07	0.13	0.08	0.08
N7	0.08	0.06	0.11	0.12
N8	0.11	0.12	0.13	0.13
H1	0.98	1.05	0.26	1.18
H2	0.88	0.95	0.28	1.09
H3	1.1	1.13	0.25	1.08
H4	0.98	1.09	0.25	0.97
H5	0.95	0.99	0.21	1.12
H6	1.12	1.03	0.22	1.19
H7	1.21	1.25	0.23	1.23
H8	1.18	1.17	0.19	1.15

TUNEL assay for 8 uninfected sera (N) and 8 H1N1 infected sera (H). Fibroblast cell lines were seeded at $5X10^4$ cells/well in a 96 well plate and were treated with either 5% uninfected sera or 5% H1N1 infected sera for 12 hours before TUNEL assay. Values are averages of duplicate absorbance readings from TUNEL positive cells for each serum.

Table A-4. Caspase 3 activity assay for 8 uninfected and 8 H1N1 infected sera treated cultured fibroblasts

	Cell Lines			
Sera	HT1080	2fTGH	U3A	U3AR
N1	240	200	170	200
N2	330	230	270	170
N3	190	180	160	270
N4	220	190	270	210
N5	260	170	220	250
N6	260	310	260	280
N7	230	210	220	240
N8	250	230	240	230
H1	3250	3030	530	3410
H2	2890	2700	420	3010
H3	2750	2530	500	2930
H4	3680	3240	580	3860
H5	2690	2420	470	2580
H6	2710	2900	560	2670
H7	3340	3520	610	3240
H8	2960	2900	570	3070

Caspase 3 activity assay for 8 uninfected sera (N) and 8 H1N1 infected sera (H). Fibroblast cell lines were seeded at $5X10^4$ cells/well in a 96 well plate and were treated with either 5% uninfected sera or 5% H1N1 infected sera for 12 hours before Caspase 3 activity assay. Values are averages of duplicate relative fluorescent unit readings from caspase 3 active cells for each serum.

Table A-5. Trypan exclusion assay for uninfected and H1N1 infected sera on cultured fibroblasts with caspase 3 inhibitor.

	Cell Lines			
Sera	HT1080	2fTGH	U3A	U3AR
N1	0.7	0.9	1.2	0.8
N2	1.2	1.3	1.7	1.6
N3	0.9	1	1.3	2.3
N4	1.5	1.6	2.1	1.6
N5	1.1	1.2	1.5	1.8
N6	0.8	1.4	1.3	1
N7	1.4	1.9	2	2.1
N8	1.5	1.8	2.2	1.3
H1	4.8	6.2	0.9	7.4
H2	5.9	7.3	0.7	6.3
H3	6.7	5.8	1.2	5
H4	4	6.1	1.4	6.8
H5	7.2	6.8	0.8	6.1
H6	7.5	5.6	1.4	5.7
H7	6.3	5.4	1.3	6.5
H8	5.6	4.6	0.8	4.2

Trypan blue exclusion assay for 8 uninfected sera (N) and 8 H1N1 infected sera (H) treated with caspase 3 inhibitor. Fibroblast cell lines were seeded at $5X10^4$ cells/well in a 96 well plate and were treated with either 5% uninfected sera or 5% H1N1 infected sera and 10 μM caspase 3 inhibitor Ac-DEVD-CHO for 12 hours before the trypan blue assay. Values are averages for percent blue staining cells for each serum.

APPROVAL FOR CONDUCTING RESEARCH INVOLVING HUMAN SUBJECTS
Research Ethics Board – Laurentian University

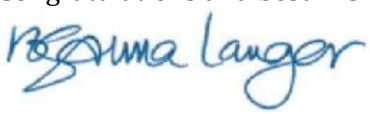
This letter confirms that the research project identified below has successfully passed the ethics review by the Laurentian University Research Ethics Board (REB). Your ethics approval date, other milestone dates, and any special conditions for your project are indicated below.

TYPE OF APPROVAL / New X / Modifications to project / Time extension	
Name of Principal Investigator and school/department	Paul Michael, Biomolecular Sciences Ph.D. program, TC Tai, Medical Sciences Division, Aseem Kumar, Chemistry and Biochemistry
Title of Project	*Cytokine Response in Septic Shock*
REB file number	2014-08-04
Date of original approval of project	November 30, 2014
Date of approval of project modifications or extension *(if applicable)*	
Final/Interim report due on: *(You may request an extension)*	November 30, 2015
Conditions placed on project	

During the course of your research, no deviations from, or changes to, the protocol, recruitment or consent forms may be initiated without prior written approval from the REB. If you wish to modify your research project, please refer to the Research Ethics website to complete the appropriate REB form.

All projects must submit a report to REB at least once per year. If involvement with human participants continues for longer than one year (e.g. you have not completed the objectives of the study and have not yet terminated contact with the participants, except for feedback of final results to participants), you must request an extension using the appropriate LU REB form. In all cases, please ensure that your research complies with Tri-Council Policy Statement (TCPS). Also please quote your REB file number on all future correspondence with the REB office.

Congratulations and best wishes in conducting your research.



Rosanna Langer, PHD, Chair, *Laurentian University Research Ethics Board*

APPROVAL FOR CONDUCTING RESEARCH INVOLVING HUMAN SUBJECTS
Research Ethics Board – Laurentian University

This letter confirms that the research project identified below has successfully passed the ethics review by the Laurentian University Research Ethics Board (REB). Your ethics approval date, other milestone dates, and any special conditions for your project are indicated below.

TYPE OF APPROVAL / New X / Modifications to project / Time extension	
Name of Principal Investigator and school/department	Paul Michael, Biomolecular Sciences Ph.D. Program, TC Tai, Medical Sciences Division Aseem Kumar, Chemistry and Biochemistry
Title of Project	Severe Pandemic H1N1 infection in I.C.U.: Comparative Resource Utilization and Pathological Disease Progression
REB file number	2014-08-05
Date of original approval of project	November 30, 2014
Date of approval of project modifications or extension *(if applicable)*	
Final/Interim report due on: *(You may request an extension)*	November 30, 2015
Conditions placed on project	

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Congratulations and best wishes in conducting your research.

Rosanna Langer, PHD, Chair, *Laurentian University Research Ethics Board*